Research Report

Expression and cell distribution of receptor for advanced glycation end-products in the rat cortex following experimental subarachnoid hemorrhage

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Article history:
Accepted 20 November 2013
Available online 26 November 2013

Keywords:
Receptor for advanced glycation end-product
Nuclear factor kappa B
Subarachnoid hemorrhage
Inflammation

Abstract

Convincing evidence indicates that inflammation contributes to the adverse prognosis of subarachnoid hemorrhage (SAH). Some pro-inflammatory molecules such as high mobility group protein 1, S100 family of proteins, β-amyloid peptide, and macrophage antigen complex 1 have been involved in the damaging inflammation process following SAH. The receptor for advanced glycation end-products (RAGE) is a transmembrane receptor that senses these molecules and plays central role in inflammatory processes. This study aimed to determine the expression and cell distribution of RAGE in the brain cortex after SAH. Male Sprague-Dawley rats were randomly divided into sham group and SAH groups at 6 h, 12 h and on day 1, day 2 and day 3 (n=6 for each subgroup). SAH groups suffered experimental SAH by injection of 0.3 ml autologous blood into the prechiasmatic cistern. RAGE expression was measured by Western blot, real-time PCR, immunohistochemistry and immunofluorescence. Nuclear expression of p65 protein, the major subunit of nuclear factor kappa B, was also detected. Our data demonstrated that the expression levels of RAGE and nuclear p65 protein were both markedly increased after SAH. Moreover, there was a significant positive correlation between the expression of RAGE and that of p65 protein. Double immunofluorescence staining showed that RAGE was expressed by neuron and microglia rather than astrocyte after SAH. These results suggest that RAGE may be directly involved in the inflammatory response after SAH, and there might be important

Abbreviations: SAH, subarachnoid hemorrhage; RAGE, receptor for advanced glycation end-products; NF-κB, nuclear factor kappa B; CNS, central nervous system; NeuN, neuron specific nuclear protein; Iba1, ionized calcium binding adaptor molecule 1; GFAP, glial fibrillary acidic protein; HMGB1, high mobility group protein 1; Aβ, β-amyloid peptide; Mac-1, macrophage antigen complex 1; PCR, polymerase chain reaction

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http://dx.doi.org/10.1016/j.brainres.2013.11.023
1. **Introduction**

Subarachnoid hemorrhage (SAH), especially aneurysmal SAH, is a fatal disease of central nervous system (CNS). Although accounting for only 5% of all strokes, SAH imposes a significant burden on society and economy, as it affects mainly middle-aged patients, leading to high mortality and disability rates (Venti, 2012). Early and delayed brain injury after SAH have been well documented, but the underlying mechanisms have not been well elucidated. Numerous findings have highlighted a strong contribution of inflammation to the early brain injury after SAH (Fassbender et al., 2001; Pradilla et al., 2010; Sehba et al., 2011). The transcription factor nuclear factor kappa B (NF-κB), which plays a crucial role in regulating the immunity and inflammation in CNS, has been reported to be activated after SAH (You et al., 2012, 2013; Zhou et al., 2007). Early activation of NF-κB upregulates the expression of pro-inflammatory cytokines, chemokines, and cell adhesion molecules that lead to the breakage of blood brain barrier, brain edema, neuronal cell apoptosis and death, resulting in poor clinical outcomes of SAH patients (Fassbender et al., 2001; Larysz-Brysz et al., 2012; Pradilla et al., 2010; You et al., 2013).

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily that can interact with and be activated by diverse pro-inflammatory ligands, including high mobility group protein 1 (HMGB1), S100 family of proteins, β-amyloid peptide (Aβ), macrophage antigen complex 1 (Mac-1, also known as complement receptor 3 or integrin CD11b/CD18) and advanced oxidation protein products (AOPPs) (Fritz, 2011; Sparvero et al., 2009; Xie et al., 2013). Ligation of RAGE triggers a series of cellular signaling events, including the activation of NF-κB, leading to the production of pro-inflammatory cytokines, and causing inflammation (Lin et al., 2009). Cumulative evidence from many earlier studies reveals that RAGE is a central signaling molecule in the innate immune system and plays a crucial role in initiation and sustentation of the inflammatory response (Chavakis et al., 2004). The abnormal upregulation and activation of RAGE are involved in diverse inflammation-associated illnesses including diabetes, sterile inflammatory disease, infectious diseases, cardiovascular diseases, cancer, Alzheimer’s disease, traumatic brain injury, and ischemic stroke (Gao et al., 2012; Liliensiek et al., 2004; Muhammad et al., 2008; Schmidt et al., 2009; Sparvero et al., 2009).

In SAH studies, most of RAGE ligands, such as HMGB1, S100 proteins, Aβ and Mac-1, have been shown to be involved in the pathological process of brain injury after SAH (Kay et al., 2003; Lefranc et al., 2005; Nakahara et al., 2009; Pradilla et al., 2004). Thus, we hypothesized that RAGE, as a coreceptor for these ligands, should take a great part in the damaging inflammatory process following SAH. However, there is a paucity of studies exploring the expression of RAGE in the brain tissue after SAH. Therefore, this preliminary study aimed to investigate the changes of RAGE expression in the brain cortex in the early stage of SAH and to clarify its potential role in pathophysiology of SAH.

2. **Results**

2.1. Expression of RAGE protein in brain cortex following SAH

Western blot was performed to assess the time course of RAGE expression. The level of RAGE protein expression was low in the sham group (Fig. 2), while it increased significantly...
by 12 h after experimental SAH, peaked on day 1 and remained ascended on day 3. There was a statistically significant difference between the sham group and 12 h, day 1, day 2, and day 3 groups, respectively. Bottom: quantitative analysis of the Western blot results for RAGE. It shows that levels of RAGE protein were significantly increased after SAH compared with the sham group. Bars represent the mean ± SD. *p < 0.01 compared with sham group.

Fig. 2 – The expression of RAGE protein in the rat brain cortex in the sham and SAH groups at various time points (n=6, each group). Top: representative autoradiogram of RAGE expression in the cortex of the brain after SAH. Lane 1, sham; lanes 2, 3, 4, 5 and 6 represent 6 h, 12 h, day 1, day 2 and day 3 groups, respectively. Bottom: quantitative analysis of the Western blot results for RAGE. It shows that levels of RAGE protein were significantly increased after SAH compared with the sham group. Bars represent the mean ± SD. *p < 0.01 compared with sham group.

Fig. 3 – Quantitative analysis of real-time PCR results for RAGE mRNA expression in the rat brain cortex. Lane 1, sham; lanes 2, 3, 4, 5 and 6 represent the 6 h, 12 h, day 1, day 2 and day 3 groups, respectively. It shows that RAGE mRNA levels in 6 h, 12 h, day 1, and day 2 groups were significantly higher than in sham group. Bars represent the mean ± SD (n=6, each group). *p < 0.01 compared with sham group.

2.2. RAGE mRNA level in the brain cortex after SAH

The expression of RAGE mRNA was tested by quantitative real-time polymerase chain reaction (PCR). As shown in Fig. 3, RAGE mRNA level was significantly increased in as early as 6 h after SAH in comparison with the sham group, and reached the peak at 12 h after SAH. There was a significant difference between the sham group and 6 h, 12 h, day 1, day 2 group (p<0.01, respectively) (Fig. 3).

2.3. RAGE immunohistochemistry after SAH

Immunohistochemistry for RAGE was performed to assess the localization of RAGE expression. RAGE was constitutively and weakly expressed in the cortex of the normal rat brain (Fig. 5, sham). While, RAGE expression was obviously increased in the cortex at day 1 after SAH (Fig. 5, SAH). Both cytosolic and nucleic immunoreactivities of RAGE in lots of neural cells were enhanced after SAH (Fig. 5, SAH).

2.4. Double immunofluorescence staining for RAGE and neural cell markers

To identify in which kind of brain cells RAGE mainly expressed after SAH, double immunofluorescence staining was performed for RAGE and neuronspecific nuclear protein (NeuN), ionized calcium binding adapter molecule 1 (Iba1) or glial fibrillary acidic protein (GFAP). As shown in Fig. 6, compared with the sham group, enhanced accumulation of RAGE was observed in NeuN-positive cells after SAH. RAGE was expressed by most of the neurons both in the sham and 24 h post-SAH groups. As Iba1 can stain both resting and activated microglia, abundant activated Iba1-positive microglia were detected in the cortex after SAH (Fig. 7). RAGE expression in microglia was increased obviously in the SAH group (Fig. 7). Merged images both in the sham and 24 h post-SAH groups showed that almost all of the Iba1-positive cells expressed RAGE. Fig. 8 showed that the number of cells positive for RAGE or GFAP was increased in SAH group. However, GFAP-positive cells that also were positive for RAGE cannot be found in the overlapped images. These results suggested that RAGE was expressed in neuron and microglia rather than astrocyte in the rat cortex after SAH.
2.5. Nuclear p65 expression in brain cortex following SAH

To investigate the NF-κB activation, nuclear expression of p65 protein, a major subunit of NF-κB, was detected by Western blot. Our data demonstrated that the expression of p65 protein was significantly increased by 6 h after SAH, peaked on day 1 and remained elevated at day 3 post-SAH (p < 0.01) (Fig. 4). There is significant positive relationship between the expression of p65 protein and that of RAGE (r = 0.721, and p < 0.0001).

3. Discussion

In this study, we reported the upregulated protein and gene expression of RAGE in the brain cortex near the subarachnoid blood clot after experimental SAH. The over-expressed RAGE could be found in neurons and microglia rather than astrocytes. Meanwhile, the nuclear expression of p65 protein, the major subunit of NF-κB, was also dramatically increased, which indicated the activation of NF-κB. There was a significant positive correlation between the time course of RAGE
expression and that of nuclear p65 protein expression. Our results suggest that RAGE might play a role in triggering and regulating the NF-κB activity and the accompanied inflammation in the early stage of SAH, and thereby contribute to the damage process of SAH.

3.1. Increased RAGE expression and elevated NF-κB activation in rat cortex after SAH

Our previous studies have established that early activated NF-κB upregulate the expressions of cytokines and adhesion molecules, which ultimately lead to brain injury after SAH (You et al., 2013; Zhou et al., 2007). Here, we aimed to testify the hypothesis that RAGE, as a coreceptor for many pro-inflammatory molecules, is also activated in the brain cortex and might play a role in the damaging inflammatory response following SAH. We eventually found that both RAGE and nuclear p65 protein expressions were dramatically increased and there was significant positive correlation between the elevations. These results are compatible with our previous findings and add to the evidence that increased RAGE expression might be involved in the damaging inflammatory response after SAH by initiating and sustaining the activation of NF-κB.

The expression of RAGE itself is also controlled by NF-κB transcription factors (Yan et al., 1994); activation of the RAGE–NF-κB signaling pathway results in an increased cell expression of RAGE (Lin et al., 2009). Meanwhile, RAGE becomes upregulated in environments rich in its ligands (Schmidt et al., 2001). Noteworthy, most of RAGE ligands, such as HMGB1, S100 proteins, Aβ and Mac-1 have been shown to accumulate in the brain or cerebrospinal fluid after SAH (Kay et al., 2003; Lefranc et al., 2005; Nakahara et al., 2009; Pradilla et al., 2004). These facts can explain the reason why RAGE expression dramatically increased after SAH.

Fig. 7 – Representative photomicrographs showed brain cortex double immunofluorescent staining for RAGE (red) and Iba-1, a microglia marker (green) in the sham (A–E) and day 1 (F–J) post-SAH groups. Nucleus was counterstained with DAPI (blue) in the same view in each section. (D) and (I) present merged images of RAGE (red), Iba-1 (green) and DAPI (blue). Compared with the sham group, the SAH group showed abundant Iba-1 positive ameboid microglia in the cortex near subarachnoid space (G). In the sham group, RAGE was weakly expressed while SAH group showed the enhanced accumulation of RAGE in both cytoplasm and nuclei. Scale bars: 50 μm.

Fig. 8 – Representative photomicrographs showed double immunofluorescent staining for RAGE (red) and GFAP, an astrocytes marker (green) in the sham (A–E) and day 1 (F–J) post-SAH groups. Nucleus was counterstained with DAPI (blue) in the same view in each section. (D) and (I) present merged images of RAGE (red) and GFAP (green); (E) and (J) present merged images of RAGE (red), GFAP (green) and DAPI (blue). Compared with the sham group (A), the SAH group (F) showed increased number of RAGE-positive cells. However, GFAP-positive cells that also were positive for RAGE cannot be found in the overlapped images (I) and (J). It suggested that RAGE was not expressed in astrocytes in the rat cortex after SAH. Scale bars: 50 μm.
Interaction of RAGE with its ligands leads to the upregulation of inflammatory pathways and thereby contributes to the inflammation-mediated brain injury after SAH. However, further investigations using RAGE special inhibitors (Deane et al., 2012) in SAH models are warranted to confirm this conclusion.

3.2. Neural cellular localization of RAGE after SAH

RAGE is mainly expressed in tissues and cell types that are critical for immune surveillance including lung, liver, vascular endothelium, monocytes/macrophages, dendritic cells, and neurons (Lin et al., 2009). In ischemic brain injury models, inflammatory response after stroke showed to be not restricted to immune cells but also to neuron cells (Tang et al., 2007) and in previous SAH researches, it was found that activated neurons after SAH contribute to the inflammation-associated brain injury (Li et al., 2012; Sun et al, 2013). Here, we showed that most of the NeuN-positive cells were also positive for RAGE, and the accumulation of RAGE in NeuN-positive cells were increased after SAH. These evidences indicate that elevated RAGE in activated neurons might be involved in the inflammation associated brain damaging process following SAH. Microglia, the resident innate immune cells in the brain, are well-known contributor to brain damage in several neurological disorders (Block et al., 2007). Previous studies have revealed that RAGE is mostly expressed by microglia and overexpression of RAGE in microglia was shown to exacerbate neuroinflammation and to increase levels of IL-1 and TNF-α in the cerebral cortex (Fang et al., 2010). Our results illustrated that the number of activated microglia was significantly increased and the accumulation of RAGE in microglia was obviously enhanced after SAH. It suggests that activation of RAGE expressed in SAH-induced active microglia could contribute to neural damage through enhancing neuroinflammation.

It should be noted that we could not find GFAP-positive astrocytes that also be stained with RAGE antibody in the rat cortex either in the sham group or in the SAH group. This result was consistent with Ma et al.’s (2003) work, in which they found that no RAGE was located in the hippocampus astrocytes in a moderate hypoxic-ischemic rat model. While, Ponath et al. (2007) and Muhammad et al. (2008) demonstrated that RAGE can be expressed by cultured mice astrocytes or by astrocytes in the mice brain. Some other studies reported that RAGE was co-localized with astrocytes in human brain with neurodegenerative disease (Ma and Nicholson, 2004; Sasaki et al., 2001; Sasaki et al., 2002). The reason for the discrepancy between our result and the previously published works is unclear at this time. One possible explanation could be that RAGE was expressed by astrocytes in brains of human and mice rather than rat. However, additional experiments are needed to confirm this conjecture.

3.3. The implication for therapeutic potential of RAGE in the brain injury after SAH

As described above, RAGE is a common and essential receptor for several pro-inflammatory molecules and may play an important role in the damaging inflammatory response following SAH. Therefore, inhibition of RAGE would perhaps be more potent to attenuate inflammatory response and early brain injury than blocking single pro-inflammatory molecule. However, the therapeutic potential of RAGE modulation to regulate the inflammation-associated early brain injury following SAH has not been previously investigated. Further studies are needed to assess the therapeutic potential of RAGE in SAH.

3.4. Summary

In this study, we reported that both RAGE protein and mRNA expressions were significantly increased after SAH. Upregulated RAGE expressions were found in neurons and microglia rather than astrocytes in the cortex of rat. Although our results are still preliminary, they provide important insight into SAH pathophysiology and the changes of RAGE expression in the early stage of SAH. It could be postulated that RAGE might play an important role in the inflammation-mediated early brain injury after SAH. However, the full role of RAGE and its therapeutic potential in SAH need further investigation.

4. Experimental procedures

4.1. Animal preparation

Male Sprague-Dawley rats (280–320 g) were purchased from animal center of Jinjing Hospital. The rats were raised on a 12 h dark-light cycle circumstance with free access to food and water. All procedures were approved by the Animal Care and Use Committee of Nanjing University and accorded to Guide for the Care and Use of Laboratory Animals by National Institutes of Health.

4.2. Animal model of SAH

Experimental SAH model was produced using stereotaxic insertion of a needle with a rounded tip and a side hole into the prechiasmatic cistern according to our previous study (Sun et al., 2013). Briefly, after intraperitoneal anesthesia with pentobarbital sodium (50 mg/kg) (Sigma, Shanghai, China), the rats were positioned prone in a stereotactic frame. The amount of 0.3 ml non-heparinized fresh autologous arterial blood was slowly injected into the prechiasmatic cistern for 20 s with a syringe pump under aseptic technique. The animals were allowed to recover 45 min after SAH. One milliliter of 0.9% NaCl solution was injected subcutaneously right after the operation to prevent dehydration. After operation procedures, the rats were then returned to their cages and the room temperature kept at 23 ± 1°C.

A total of 70 male rats were used in our study. The mortality rate of rats in the sham group was 0% (0/18 rats), and it was 19% (10/52 rats) in the SAH group. Thirty rats with SAH were randomly divided into five subgroups and killed by ventricle perfusion at 6, 12 h, and on day 1, day 2, day 3 post-SAH, (n=6 for each subgroup). Another 12 rats with SAH were selected randomly for immunohistochemistry and immunofluorescence study of day 1 group (n=6). Sham animals
experienced the same surgery process except for injection with anything into the prechiasmatic cistern. In our pilot study, we found that there was no statistical difference of all detected variables among sham groups at each time point (data not shown). Therefore, animals in sham group were sacrificed at 24 h after sham operation.

4.3. Perfusion-fixation and tissue preparation

The rats were anesthetized as above, and perfused through the left cardiac ventricle with 0.9% normal saline solutions until the effluent from the right atrium was clear. Animals which had obvious blood clots in the prechiasmatic cistern were selected for further analysis. After blood clots on the brain tissue were cleared carefully, the temporal lobe tissue (see Fig. 1) was harvested on ice and stored in −80 °C for Western blot and real-time PCR analysis. For immunohistochemistry and immunofluorescence study, the rats were perfused with 0.9% NaCl solutions followed by 4% buffered paraformaldehyde; the brain was immersed in 4% buffered paraformaldehyde overnight and then embedded in paraffin for immunohistochemistry study while frozen in OCT for immunofluorescence study.

4.4. Total/nuclear protein extraction

To extract cortex total protein, proper size of tissues were mechanically lysed in 20 mM Tris (pH 7.6), which contained 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.11 IU/ml aprotinin (all from Sigma, Shanghai, China). Homogenates were centrifuged at 14,000 g for 15 min. The supernatant containing nuclear proteins was collected and stored at −80 °C until analysis.

Cortex nuclear protein was extracted following the previous studies (Li et al., 2011; Zhou et al., 2007). Briefly, 100 mg of fresh cortex was homogenized in 0.8 ml of iced-cold buffer A, which consisted of 10 mM HEPES (pH 7.9), 2 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.5 mM PMSF (all from Sigma, Shanghai, China). Then 30 μL Nonidet P-40 was added to the system. After centrifugation at 4 °C for 30 min, the supernatant (cytoplasmic fraction) and resuspended the nuclear pellet with 200 μL buffer B, which contained 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 25% (v/v) glycerol. The mixture was centrifuged at 14,000 g at 4 °C for 15 min. The supernatant containing nuclear proteins was collected and stored at −80 °C until analysis.

4.5. Western blot analysis

Protein concentrations of the supernatant were determined using the BCA method. Equal protein amounts per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene-difluoride (PVDF) membrane. The membranes were blocked in blocking buffer (5% skimmed milk in TBS containing 0.05% Tween 20 (TBST)) for a minimum of 1 h, and incubated overnight at 4 °C with primary rabbit antibodies against RAGE (1:1000, Abcam, USA) and GAPDH (1:3000, Bioworld, USA) in TBST containing 1% skimmed milk. For detection of nuclear p65 protein, we used primary rabbit anti-p65 antibody (1:200, Santa Cruz Biotechnology, USA) and primary rabbit antibody against Histone 3 (1:1000, Cell Signaling Technology, USA) as internal reference. After being washed with TBST (3 × 15 min), the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (1:3000, Santa Cruz Biotechnology, USA) for 2 h at room temperature. The blotted protein bands were visualized by enhanced chemiluminescence (Thermo Scientific, USA) and were exposed to X-ray film. Relative changes in protein expression were estimated from the mean pixel density using UN-SCAN-IT 6.1 software (Silk Scientific Inc., USA).

4.6. RNA isolation and quantitative real-time PCR

Temporal lobe tissues were prepared according to the RNA extraction protocol. Total RNA was extracted from Temporal lobe cortex samples using RNAiso Plus (TaKaRa Bio, Dalian, China). The concentration and purity of total RNA were determined by spectrophotometer (OD260/280, 1.8–2.2) and 1% agarose gel electrophoresis. In order to avoid RNA degragation, part of the RNA was reverse transcribed to cDNA immediately using PrimeScript™ RT reagent Kit (TaKaRa Bio, Dalian, China), and the surplus RNA was kept at −80 °C. The primers were designed according to PubMed GenBank, and synthesized by Invitrogen Life Technologies (Shanghai, China). The primer sequences were as follows: RAGE: F: 5'-AACCTACCGAAATCCGATCA-3', R: 5'-ACAATCTGTCCTTGGCCCATCA-3'; GAPDH: F: 5'-ACACGCAACAGGTTGTGGA C-3', R: 5'-TGGAGGGTGCCCGAAACTT-3'. Quantitative real-time PCR analysis was performed using the Agilent Technologies Stratagene Mx3000P real-time PCR system (Genetimes Technology Inc., Shanghai, China), applying real-time SYBR Green PCR technology. The reaction mixtures contained 1 μL cDNA, 12.5 μL SYBR Green (TaKaRa Bio, Dalian, China), 1 μL of each forward and reverse primer (10 μM) and nuclease-free water to a final volume of 25 μL. PCR amplification program consisted of an initial denaturation step of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, a 30 s annealing step at 65 °C, and a 30 s extension step at 75 °C. Fluorescent readings were taken during the extension step of each cycle. Melting curve analysis was also performed to ensure the amplification of a single PCR product. All samples were analyzed in triplicate. GAPDH was selected as an acceptable endogenous reference housekeeping gene. Relative change in RAGE mRNA expression following SAH was determined by the equation: fold change = $2^{-\frac{\Delta \Delta Ct}{\Delta \Delta Ct}}$, with ΔCt = (CtRAGE - CtGAPDH)SAH - (CtRAGE - CtGAPDH)sham (Schmittgen and Livak, 2008). Ct value is the cycle number at which fluorescence signal crosses the threshold.

4.7. Immunohistochemical staining

The tissue was fixed with the 4% paraformaldehyde and embedded in paraffin. Immunohistochemical staining was performed as our previous study (Sun et al., 2013). Detailedly, the tissue sections (2 μm) were used for immunohistochemical staining; the sections were deparaffinized as usual and incubated with 3% H₂O₂ in phosphate-buffered saline (PBS) for 10 min. Sections were blocked with 5% normal fetal
bovine serum in PBS for 2 h followed by incubation with anti-RAGE antibody (1:100, Abcam, USA). After washing carefully for half an hour, each of the sections was incubated with HRP-conjugated goat anti-rabbit IgG (1:500, Santa Cruz, USA) for 60 min at room temperature. After washing for half an hour again, diaminobenzidine was used as a chromogen and counterstaining was done with hematoxylin. The negative control was also performed without adding RAGE antibody, and the other steps were the same between the experiment sections and negative control.

4.8. Immunoﬂuorescence staining

Immunoﬂuorescence staining was performed according to our previous study in our laboratory (Sun et al., 2013). Brain tissue was ﬁxed with 4% paraformaldehyde overnight and dipped in 20% saccharose PBS for 2 days and then in 30% saccharose PBS for another 2 days to remove water in the tissue. Sections 6 μm in thickness were sliced and blocked with 5% normal fetal bovine serum in PBS containing 0.1% Triton X-100 for 1 h at room temperature prior to incubation with anti-NeuN antibody (1:200, Millipore, USA) and anti-RAGE antibody (1:100, Abcam, USA) or anti-β1 antibody (1:200, Abcam, USA) and anti-RAGE antibody (1:100, Abcam, USA) or anti-GFAP antibody (1:200, BD Biosciences, USA) and anti-RAGE anti-body (1:100, Abcam, USA) overnight at 4°C. After sections were washed three times with PBS for 45 min, they were incubated with proper secondary antibodies (Alexa Fluor 488 and Alexa Fluor 594, 1:200) for 2 h at room temperature. The slides were washed with PBS again three times for 45 min prior to be counterstained by DAPI for 2 min. After three washes again, the slides were covered by microscopic glass with Anti-fade Mounting Medium for further study. Negative controls were prepared by omitting the primary antibodies. Fluorescence microscopy imaging was performed using ZEISS HB050 inverted microscope system and handled by Image-Pro Plus 6.0 software (Media Cybernetics, USA) and Adobe Photoshop CS5 (Adobe Systems, USA).

4.9. Statistical analysis

SPSS 17.0 was used for the statistical analysis (SPSS, Inc., Chicago, USA). All data were presented as mean ± SD. The data were subjected to one-way ANOVA followed by Tukey’s post hoc test. The relationship between RAGE protein expression and nuclear p65 expression was analyzed using the linear regression model. Statistical signiﬁcance was deﬁned as p < 0.05.

Acknowledgments

The authors acknowledge the support for this study by Grants from the National Natural Science Foundation, China (No. 81171170) and Nature Science Foundation of Jiangsu Province, China (BK2010459) and Scientiﬁc Research and Innovation Foundation of Universities in Jiangsu Province, China (CXZZ12_0067).


