RAGE expression is up-regulated in human cerebral ischemia and pMCAO rats

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ABSTRACT

To determine whether the receptor for advanced glycation endproducts (RAGE) contributes to cerebral ischemia, we evaluated RAGE expression in human cerebral ischemia and a model of permanent middle cerebral artery occlusion (pMCAO) in rats. Biopsy specimens were obtained from 12 patients with unilateral cerebral infarction. For the pMCAO model, the middle cerebral artery (MCA) of Sprague–Dawley (SD) rats was permanently occluded. Immunohistochemistry and Western blotting were used to measure RAGE expression in the ischemic hemisphere relative to the normal hemisphere. PC12 cells subjected to oxygen and glucose deprivation (OGD) were used to evaluate the role of RAGE in cell injury. As expected, cerebral ischemia patients expressed elevated levels of RAGE in the ischemic hemisphere. In 1 and 2 days pMCAO rats, levels of RAGE were higher in the ischemic hemisphere relative to the non-ischemic hemisphere, and expression was primarily located in the penumbra of the ischemic hemisphere. In PC12 cells, levels of RAGE increased after 7 h of OGD culture. Notably, blockade of RAGE with a selective RAGE antibody *in vitro* reduced the cytotoxicity caused by OGD. The present data suggest that RAGE is up-regulated in human cerebral ischemia and pMCAO rats, suggesting a role for RAGE in brain ischemia.

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Cerebral ischemia is associated with robust inflammatory responses and neuronal loss, and may lead to brain malfunction and death. The receptor for advanced glycation endproducts (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules that can interact with various molecules, including β-amyloid peptide (Aβ), S100B, high-mobility group-box protein 1 (HMGB1) and advanced glycation endproducts (AGE) [7,8,15,23]. In the inflammatory events in Alzheimer's disease (AD), RAGE is involved in microglial activation [12] and mediates the infiltration of inflammatory cells across the blood brain barrier (BBB) [3]. Furthermore, the four primary ligands of RAGE have all been shown to be involved in cerebral ischemia [4,9,17,25]. Some RAGE ligands, such as AGE and HMGB1, mediate microglial activation and neurotoxicity contributing to ischemic injury [9,25]. However, RAGE is not the only receptor that binds these ligands [19,21]. Thus, it remains unknown whether RAGE mediates the inflammatory cascades induced by these ligands following cerebral ischemia in human and pMCAO rats.

In the present study, we evaluated the expression of RAGE in human cerebral ischemia biopsies as well as in a rat model of cerebral ischemia, permanent middle cerebral artery occlusion (pMCAO). In addition, we explored the mechanism involved in ischemia-induced alterations in RAGE expression through OGD treatment in PC12 cells.

Samples were provided by the Neurology Department of the First Clinical College of Harbin Medical University. The deaths of the patients were caused by cerebral ischemic infarction and diagnosed by computerized tomography. Twelve cases were studied, with an average age of 61.4 (ranging from 43 to 68). Tissue samples were taken from the area adjacent to the infarct site and same area of the normal hemisphere of each human brain sample. The tissue was routinely embedded in paraffin and cut into sections (10 μm). Slides were divided into three groups according to duration between stroke and death: <2-day group, 3–5-day group, and the >5-day group.

The animal studies were approved by Harbin Veterinarian Research of the Agricultural Ministry, China, and were implemented according to the guidelines published by the National Institute of Health. Male Sprague–Dawley rats weighing 250–300 g were used for the pMCAO models according to the procedure described previously [11]. Twenty rats were divided into five groups according to time from ischemia to euthanasia (12 h, 24 h, 2 days, 6 days, and sham group, $n = 4$/group). The sham group was treated in the same way except for the middle cerebral artery occlusion and the rats were sacrificed 24 h after the operation and brains were
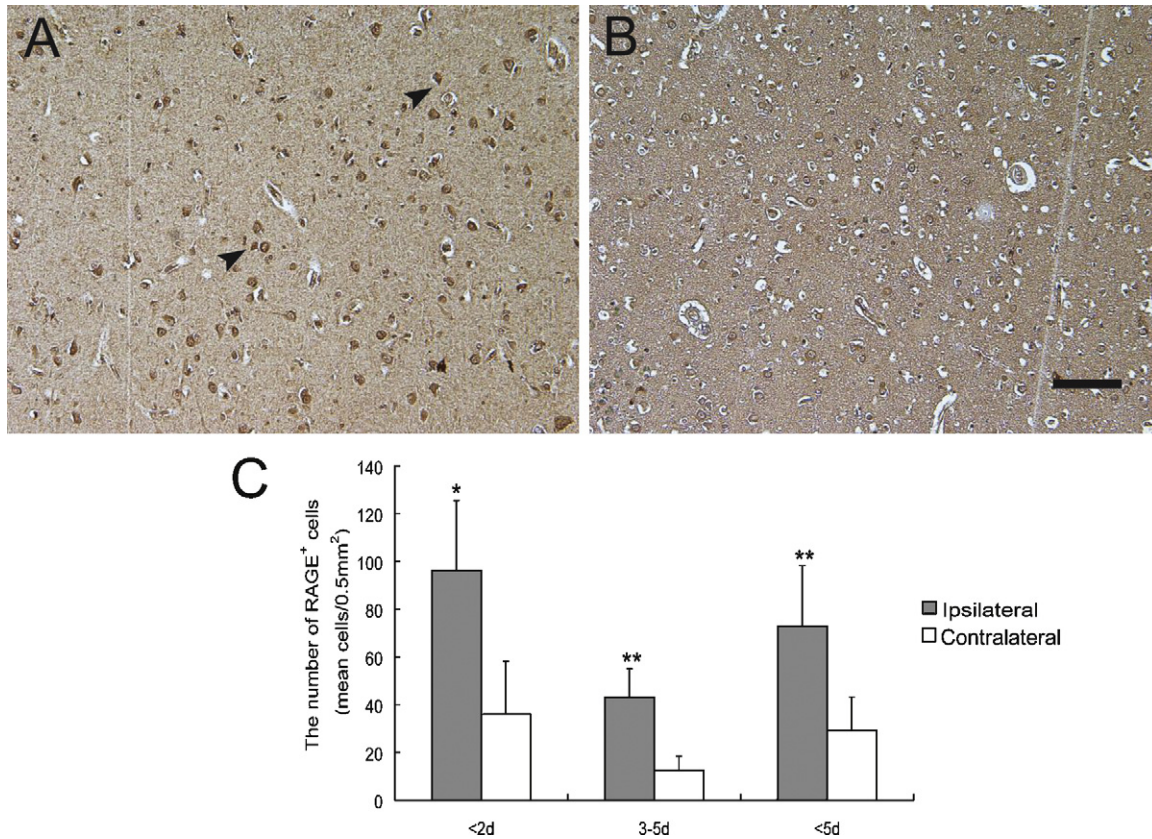
Fig. 1. Expression of RAGE in human cerebral ischemia brain sections. The RAGE$^+$ cells were stained brown in the ischemic hemispheres (*arrows*) (A) but fewer in the non-ischemic hemispheres (B) (*scale bar* = 50 μm). The differences between the ischemic and non-ischemic hemispheres were significant in each group (C). (*$P < 0.05$, **$P < 0.01$, $n = 4$, compared with the corresponding values in the contralateral hemispheres).

quickly removed. Coronal brain sections were taken from bregma 0.7 mm to bregma −3.3 mm, according to the stereotaxic coordinates [5]. Tissue was routinely fixed and embedded with paraffin. Serial sections were made and processed for hematoxylin–eosin (H.E.) staining or immunohistochemistry.

The ipsilateral cortex of pMCAO rats was homogenized and lysed with cell lysis buffer (P0013, Beyotime, China) according to the manufacture's protocol. Samples were run on 12% SDS–PAGE electrophoresis. The Bradford method was used to ensure equal protein loading. After electrophoresis, the protein was transferred to PVDF membranes for 1 h. The membrane was blocked with 5% non-fat milk for 2 h at RT and blotted with rabbit anti-RAGE antibody (1:200 diluted, Chemi-Con, USA) at 4 °C overnight, and detected by a chemiluminescence kit (W3960, Promega, USA) using anti-rabbit IgG AP conjugated secondary antibody (1:1000). β-Actin (1:1000 diluted, Santa Cruz, USA) was used as a reference.

For the immunohistochemistry, both human cerebral ischemia and pMCAO rat brain sections were de-waxed, fixed, and blocked according to standard procedures, then incubated with anti-RAGE antibody (1:200, Chemi-Con, USA) overnight at 4 °C. After rinsing, the sections were incubated with a biotinylated rabbit anti-goat antibody (1:500, Zhongshan Golden Bridge, China) at 37 °C for 30 min, followed by avidin–biotin complex (Zhongshan Golden Bridge, China) for 20 min at RT. After rinsing, 3,3′-diaminobenzidine (DAB) was applied to the sections. Reaction with non-immune IgG of the relevant species served as negative controls. The total number of RAGE positive cells was counted in the entire ischemic region for each section. The number of positive cells per 0.5 mm^2 of tissue area was calculated.

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO$_2$ humidified incubator (SANYO, Japan). For oxygen and glucose deprivation (OGD), the cells were then incubated in glucose-free DMEM (Invitrogen, USA) in an oxygen-free chamber with 95% N$_2$ and 5% CO$_2$ for 12, 20 or 30 h for the LDH and flow cytometry assay. An anti-RAGE antibody (5 μg/ml, Biosynthesis, China) was used to neutralize RAGE activity. For Western blotting, the OGD culturing time was 7 or 12 h. A post-ischemia state was created by adding glucose (to a concentration of 4.5 mg/ml) and returning the culture to a normal incubator for overnight incubation (12 h). Normal culture medium was added to the control groups at the same volume in order to maintain conformity, and control cells were incubated in a normal incubator.

For the lactate dehydrogenase (LDH) assay, PC12 cells were subjected to OGD culture as described above. After various treatments, the total cellular LDH was detected using LDH Detection Kit (Jiancheng, China) following the manufacture's protocol. Each sample was run in triplicate. The measurement was carried out using a UV-spectrometer (Purkinjie General Instrument, China) at 440 nm.

For the flow cytometry analysis, PC12 cells were cultured in OGD as described above and collected. After resuspension with 1 ml PBS, a 100 μl sample from each group was incubated in 500 μl binding buffer and 5 μl Annexin V-PE (BioVision, USA) for 15 min at RT in the dark. Cell mortality was analyzed by flow cytometry (FACSCalibur, BD Bioscience, USA).

Values are presented as mean ± S.D. Comparisons were performed using the *t*-test or one-way ANOVA with Newman–Keul's test. Differences were considered significant when $P < 0.05$.
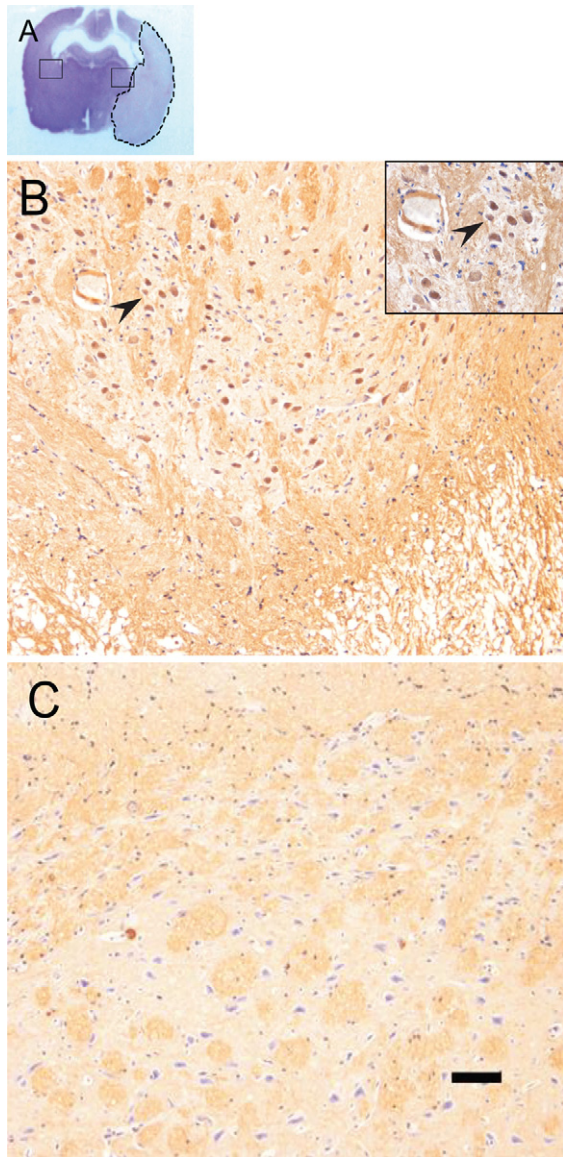
Fig. 2. Expression of RAGE in pMCAO rat brains. (A) Hematoxylin-eosin (H.E) stain-ing of a representative section, the infarct area is indicated with dashed lines. The boxes indicate the same area of the ischemic and normal hemispheres where higher resolution analysis was performed for Fig. 2B and C. (B) The RAGE$^+$ cells were stained brown (*arrow*). (C) The negative control hemisphere contained few RAGE$^+$ cells (*scale bar* = 100 μm).

Fig. 3. Western blotting analysis of RAGE expression in pMCAO rat brains. RAGE expression was elevated after 1–2 days of ischemic injury (*$P < 0.05$, compared with the sham operation group, $n = 4$).

neuronal pyknosis, karyolysis, and decreased eosin staining, while the penumbra area was identified from the edge of the infarction to 1 mm toward adjacent cerebral tissue [6,10,24]. The RAGE$^+$ cells of the ischemic ipsilateral hemisphere were stained brown in the penumbra. Immunohistochemical staining demonstrated that after 1 day of ischemic injury, RAGE was highly expressed in the ischemic penumbra relative to the sham operation group (Fig. 2B and C).

As shown in Fig. 3, RAGE expression in the pMCAO brain was ele-vated after 1 day of ischemic insult. After 2 days, RAGE expression was decreased relative to 1 day, but the level of RAGE remained higher than levels in the sham operation, 12 h and 6 days group ($P < 0.05$).

Western blot analysis indicated that the expression of RAGE in normal PC12 cells was lower than in the 7 and 12 h OGD groups (Fig. 4A, $P < 0.05$). Furthermore, the difference between the 7 and 12 h group was also significant (Fig. 4A, $P < 0.05$). Thus, our find-ings indicated that the expression of RAGE was elevated following OGD insult. In the LDH assay of normal control (NC) group, the LDH concentration was 56.44 ± 10.50 μmol/l versus 50.70 ± 8.12 μmol/l in the non-blockade group and RAGE blockade group, respectively (Fig. 4B). In the 12, 20 and 30 h non-blockade OGD cultured groups, the LDH concentrations were 1056.27 ± 2.43, 1221.07 ± 2.42 and 1150.64 ± 4.22 μmol/l, respectively. In the 12, 20 and 30 h RAGE-blockade groups, LDH levels were 216.78 ± 1.11, 374.53 ± 2.50 and 746.60 ± 2.53 μmol/l, respectively (Fig. 4B). Thus, the concentration of LDH in the RAGE blockade group was significantly lower than in the respective non-blockade group (Fig. 4B, $P < 0.001$).

Flow cytometry analysis revealed that the cell death rate of normal PC12 cells was $0.08\% \pm 0.05\%$ versus $0.07\% \pm 0.03\%$ in the non-blockade group and RAGE blockade group, respec-tively (Fig. 4C, $P > 0.05$). In the 12 h OGD group, blocking RAGE reduced PC12 cell death rate from $1.93\% \pm 0.48\%$ to $0.57\% \pm 0.21\%$ (Fig. 4C, $P < 0.05$). In the 20 h OGD group, the cytotoxicity rate following RAGE blockade and non-blockade was $2.67\% \pm 0.87\%$ and $4.33\% \pm 1.65\%$, respectively (Fig. 4C, $P < 0.05$). When subjected to 30 h OGD insult, the cytotoxicity rate in the RAGE blockade and non-blockade groups was, respectively, $18.45\% \pm 5.95\%$ versus $25.26\% \pm 4.57\%$ (Fig. 4C, $P < 0.05$).

The interaction between RAGE and its ligands may lead to mul-tiple consequences to the immune system and neurons [12,20]. Our *in vivo* and *in vitro* studies demonstrate that (1) RAGE is up-regulated in the penumbra of the 1 and 2 days pMCAO rat brains and also in human cerebral ischemia; and (2) antibody-mediated block-ade of RAGE significantly reduces PC12 cell injury and death caused

Following immunohistochemistry, RAGE-positive (RAGE$^+$) cells were stained brown (Fig. 1). RAGE expression in the ischemic hemi-sphere was significantly greater than in the opposite non-ischemic hemisphere (Fig. 1A and B). In the <2-day group, the number of RAGE$^+$ cells was significantly higher in the ischemic hemi-sphere than the non-ischemic hemisphere (96 ± 29.3 cells/0.5 mm^2 versus 36 ± 22.2 cells/0.5 mm^2, respectively, $P < 0.05$, Fig. 1C). In the 3–5 days group, the difference in the number of RAGE$^+$ cells in the ischemic hemisphere and non-ischemic hemisphere also achieved statistical significance (42 ± 11.8 cells/0.5 mm^2 ver-sus 12 ± 5.9 cells/0.5 mm^2; respectively, $P < 0.01$, Fig. 1C) as it also did in the <5-day group (73 ± 25.3 cells/0.5 mm^2 versus 29 ± 14.0 cells/0.5 mm^2, $P < 0.01$, Fig. 1C).

H&E staining of a section adjacent to the section shown in Fig. 2B and C is shown in Fig. 2A to identify the infarct and penumbra areas. The infarction and its border were identified by
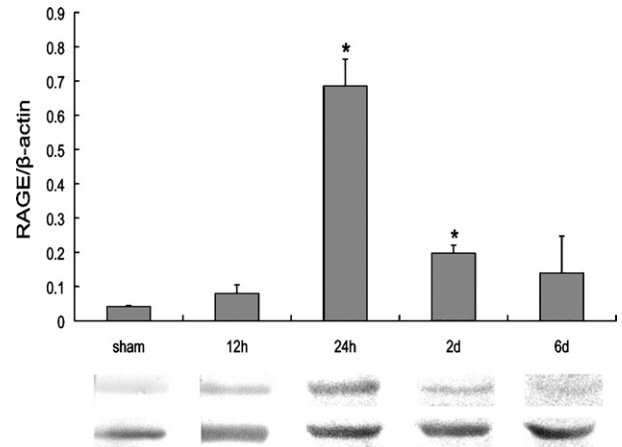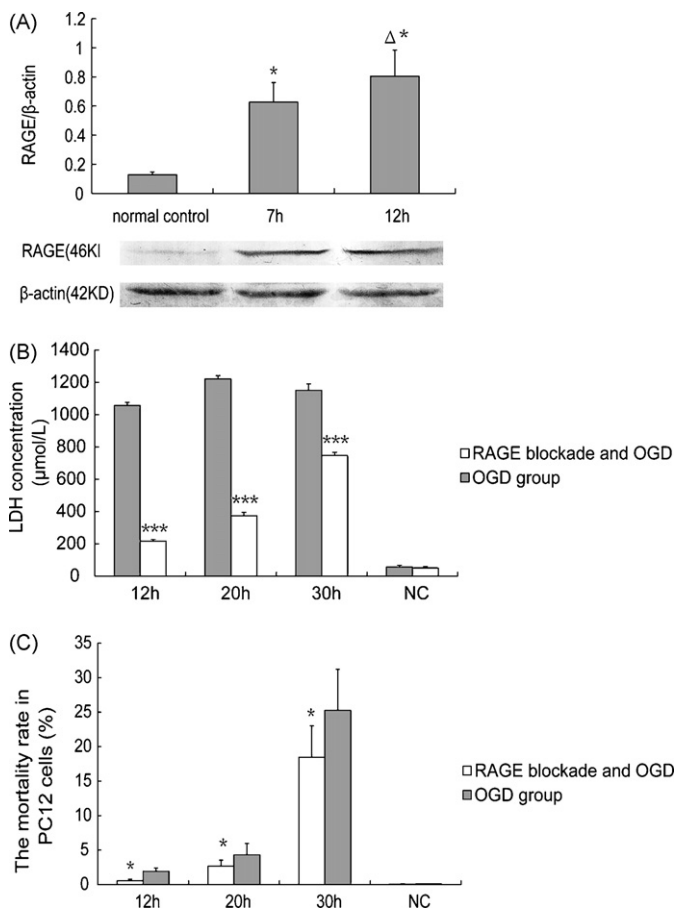
Fig. 4. Effect of RAGE in oxygen and glucose deprivation (OGD)-treated PC12 cells. (A) Expression of RAGE in oxygen and glucose deprivation (OGD)-treated PC12 cells (*$P < 0.05$, compared with normal control group, $\Delta P < 0.05$, compared with 7 h OGD group $n = 3$). (B) Lactate dehydrogenase (LDH) release by the injured PC12 cells (***$P < 0.001$, $n = 3$, compared with corresponding values in the OGD groups). (C) The cell death rate of OGD cultured PC12 cells (*$P < 0.05$, $n = 3$, compared with corresponding values in the OGD groups).

by OGD. Consistent with our findings, Ma et al. [14] found that RAGE was expressed in the injured hippocampus of the moderate hypoxic-ischemic (HI) rat brain injury model and was implicated in neuronal death. During the time of the greatest RAGE up-regulation, the ischemic brain undergoes acute inflammation, including glial activation, leukocyte infiltration, and secondary cell death [22]. The coincident timing of post-ischemic injury and RAGE up-regulation suggests that RAGE may participate in inflammation and neuronal death following ischemic injury.

To the best of our knowledge, this is the first report concerning alterations in RAGE expression in the ischemic human brain. Our findings demonstrated that RAGE participated in the human cerebral ischemic injury process between 2 to more than 5 days post-infarct. In parallel with our novel study on human ischemic tissue, Pichiule et al. [16] reported an up-regulation of RAGE expression in the hypoxic mouse brain, notably showing that expression of the RAGE gene was regulated by hypoxia inducible factor-1 (HIF-1). However, as is often the case, experimental controls with human samples are not as well-controlled as with rat models. Thus, it should not be neglected that there is considerable individual human variation. Of course, further studies are needed to increase the number of samples. Nonetheless, our results confirmed that the expression of RAGE was up-regulated in the human post-ischemic brain, providing a foundation for further studies on the role of RAGE in human cerebral ischemia.

PC12 cells grown under OGD conditions have been studied extensively as an *in vitro* model system for the mechanisms of neuronal death after ischemic insult and potential neuroprotective targets [1]. RAGE is expressed on PC12 cells [2]. In addition, an anti-RAGE antibody is widely used as an effective anti-inflammatory treatment in several diseases [13]. However, the role of RAGE in PC12 cells is not well understood. We found increased LDH release in the PC12 cells after 12, 20 and 30 h of OGD treatment. This LDH was significantly abolished by antibody-mediated RAGE inhibition. Because LDH release is a hallmark of cell injury *in vitro*, we can infer that RAGE activation is involved in OGD-induced injury to PC12 cells. This effect of RAGE activation may follow this receptor's up-regulation, as seen in the Western blotting experiments. In the present experiments, we demonstrated that RAGE was involved in OGD-induced injury and blockade of the RAGE signaling pathway could alleviate the OGD-induced injury. In RAGE-deficient mice, the infarct volume of the ischemic hemisphere was enlarged relative to wild type mice, suggesting that RAGE may be a neuroprotective factor in cerebral ischemia [16]. However, our *in vitro* study demonstrated that RAGE blockade could protect the PC12 cells from injury and death. The reason for this discrepancy is currently unknown, but may be related to differences *in vitro* and *in vivo*, or perhaps to the different way RAGE expression is blocked. From 12 to 30 h of OGD, the cytotoxicity to PC12 cells increased significantly. Taken with our Western blotting and LDH assay findings, our data suggest that cells begin to die abundantly following RAGE up-regulation and LDH release. When transthyretin (TTR) fibrils were co-cultured with RN-22 rat endothelial cells, Schwann cells and neurons, blockade of RAGE with an anti-RAGE antibody could inhibit cell death [20]. In addition, RAGE expression was closely associated with apoptosis in myoblast cell culture [18], however previous findings suggested that myoblast apoptosis stimulated by S100B was RAGE-independent [19]. In 12 and 20 h OGD groups, blockade of RAGE activity was able to reduce the rate of cell death by about one- and two-fold, respectively. However, it could only reduce cell death by 6.81% in the 30 h OGD group, indicating that additional RAGE-independent pathways are involved in ischemic cell death. Nevertheless, our results demonstrated that RAGE could facilitate the cell death in each of these three groups. These findings provide evidence to support a role for RAGE in cerebral ischemia.

In summary, the present findings demonstrate that RAGE is up-regulated in human cerebral ischemia and in the ischemic penumbra of pMCAO rats. Furthermore, RAGE mediates PC12 cell death following OGD treatment *in vitro*. Given these results, our *in vitro* and *in vivo* findings indicate that RAGE is involved in cerebral ischemia and may represent a suitable therapeutic target for the treatment of brain ischemic injury.

Acknowledgements

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