American Heart Association | American Stroke Association®

The TLR9 Ligand, CpG-ODN, Induces Protection against Cerebral Ischemia/Reperfusion Injury via Activation of PI3K/Akt Signaling

Chen Lu, MD; Tuanzhu Ha, MD; Xiaohui Wang, MS; Li Liu, MD; Xia Zhang, MD; Erinmarie Olson Kimbrough, BS; Zhanxin Sha, MS; Meijian Guan, BS; John Schweitzer, MD; John Kalbfleisch, PhD; David Williams, PhD; Chuanfu Li, MD

Background—Toll-like receptors (TLRs) have been shown to be involved in cerebral ischemia/reperfusion (I/R) injury. TLR9 is located in intracellular compartments and recognizes CpG-DNA. This study examined the effect of CpG-ODN on cerebral I/R injury.

Methods and Results—C57BL/6 mice were treated with CpG-ODN by i.p. injection 1 hour before the mice were subjected to cerebral ischemia (60 minutes) followed by reperfusion (24 hours). Scrambled-ODN served as control-ODN. Untreated mice, subjected to cerebral I/R, served as I/R control. The effect of inhibitory CpG-ODN (iCpG-ODN) on cerebral I/R injury was also examined. In addition, we examined the therapeutic effect of CpG-ODN on cerebral I/R injury by administration of CpG-ODN 15 minutes after cerebral ischemia. CpG-ODN administration significantly decreased cerebral I/R-induced infarct volume by 69.7% ($6.4\pm1.80\%$ vs $21.0\pm2.85\%$, $P<0.05$), improved neurological scores, and increased survival rate, when compared with the untreated I/R group. Therapeutic administration of CpG-ODN also significantly reduced infarct volume by 44.7% ($12.6\pm2.03\%$ vs $22.8\pm2.54\%$, $P<0.05$) compared with untreated I/R mice. Neither control-ODN, nor iCpG-ODN altered I/R-induced cerebral injury or neurological deficits. Nissl staining showed that CpG-ODN treatment preserved neuronal morphology in the ischemic hippocampus. Immunoblot showed that CpG-ODN administration increased Bcl-2 levels by 41% and attenuated I/R-increased levels of Bax and caspase-3 activity in ischemic brain tissues. Importantly, CpG-ODN treatment induced Akt and GSK-3β phosphorylation in brain tissue and cultured microglial cells. PI3K inhibition with LY294002 abolished CpG-ODN-induced protection.

Conclusion—CpG-ODN significantly reduces cerebral I/R injury via a PI3K/Akt–dependent mechanism. Our data also indicate that CpG-ODN may be useful in the therapy of cerebral I/R injury. (***J Am Heart Assoc***. 2014;3:e000629 doi: 10.1161/JAHA.113.000629)

Key Words: apoptosis • cerebral ischemia/reperfusion injury • CpG-ODN • toll-like receptors

Stroke is the third leading cause of death and the leading cause of long-term disability in the United States. About 795 000 Americans suffer a new or recurrent stroke annually.[1] Approximately 610 000 of these are first attacks and 185 000 are recurrent attacks.[1] Cerebral ischemia/reperfusion (I/R) injury (ischemic stroke) accounts for about 83% of all stroke cases.[1] At present, there is no effective treatment for cerebral I/R injury. Numerous studies have demonstrated that the innate immune and inflammatory responses mediated by Toll-like receptors (TLRs) play an important role in cerebral ischemia/reperfusion (I/R) injury.[2–4] TLRs are pattern recognition receptors that play a critical role in the induction of innate immune and inflammatory responses.[5] Recent evidence suggests that TLRs may be the important targets for development of new treatment approaches for cerebral I/R injury.[6–10] We have demonstrated that TLR4 deficiency or TLR2 modulation significantly attenuates brain injury in response to cerebral I/R.[10–12] Published data have also shown that TLR agonists attenuate cerebral I/R injury through a preconditioning mechanism.[9,13–15]

TLR9 is located intracellularly in endosomes and endoplasmic reticulum.[16] TLR9 recognizes unmethylated CpG-DNA from bacteria and endogenous DNA.[16] Synthetic CpG-oligodeoxynucleotide (ODN) has been reported to activate TLR9,[16,17] improve cell survival, prevent cell apoptosis,[18]

attenuate cardiac dysfunction after I/R[19,20] and improve outcome in shock induced by polymicrobial sepsis[21–23] or trauma hemorrhage.[24] Scholtzova et al reported that administration of CpG-ODN effectively ameliorated Alzheimer's disease-related pathophysiology.[25] Stevens et al reported pretreatment of animals with CpG-ODN for 24 hours induced neuroprotection against ischemic injury through a preconditioning mechanism.[14] Because preconditioning requires a prolonged pretreatment time, it is important to investigate whether therapeutic administration of CpG-ODN will attenuate cerebral I/R injury.

Phosphoinositide 3-kinases (PI3Ks) and their downstream target serine/threonine kinase Akt are a conserved family of signal transduction enzymes that are involved in regulating cellular activation, inflammatory responses, and apoptosis.[26] Activation of PI3K/Akt-dependent signaling plays a role in protection against organ injury in response to I/R,[20,27,28] septic shock[23] as well as trauma hemorrhage.[24] Recent evidence has also shown cross talk between PI3K/Akt signaling and TLR-mediated pathways.[20,23,29] We have previously reported that activation of PI3K/Akt signaling contributes to the protection against cerebral I/R injury by modulation of TLR2.[30]

The goal of the present study was to investigate the effect of CpG-ODN on cerebral I/R injury. We observed that CpG-ODN administration significantly reduced infarct volume and improved neurological functional recovery after cerebral I/R injury. Therapeutic administration of CpG-ODN also markedly decreased infarct volume following cerebral I/R.

Materials and Methods

Animals

Male C57BL/6 mice were purchased from The Jackson Laboratory. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments outlined in this article conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, eighth Edition, 2011). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

Focal cerebral ischemia/reperfusion

Focal cerebral I/R was induced by occlusion of the middle cerebral artery (MCAO) on the left side as described in our previous studies.[9,10,30,31] Briefly, mice (23 to 25 g body weight) were anesthetized by 5.0% Isoflurane and anesthesia was maintained by inhalation of 1.5% to 2% Isoflurane driven by 100% oxygen flow. Mice were intubated and ventilated using a rodent ventilator at a rate of 110 breaths/minute

with a total delivered volume of 0.5 mL. Body temperature was regulated at 37°C by surface water heating. Following the skin incision, the left common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) were carefully exposed. Microvascular aneurysm clips were applied to the left CCA and the ICA. A coated 6-0 filament (6023PK; Doccol Corp) was introduced into an arteriotomy hole, fed distally into the ICA. After the ICA clamp was removed, the filament was advanced 11 mm from the carotid bifurcation, and focal cerebral ischemia started. After ischemia for 60 minutes, the filament and the CCA clamp were gently removed (reperfusion starts). The collar suture at the base of the ECA stump was tightened. The skin was closed, anesthesia discontinued, and the animal allowed to recover in pre-warmed cages. Control mice underwent a neck dissection and coagulation of the external carotid artery, but no occlusion of the middle cerebral artery.

Measurement of cerebral blood flow

Successful occlusion of the middle cerebral artery was verified and recorded by laser-Doppler flowmetry (Model PeriFlux System 5000; Perimed) as described previously.[30] Briefly, under anesthesia, a midline incision of the head was made and a probe holder was attached to the skull with crazy glue at 6 mm lateral and 1 mm posterior to the bregma. A laser-Doppler probe was connected to the probe holder, and regional cerebral blood flow (CBF) was monitored and recorded. The data was retrieved continuously, stored in a computer, and analyzed using the Perimed data acquisition and analysis system. Regional CBF was expressed as a percentage of preischemic baseline values.

Experimental Design

To examine the role of CpG-ODN on focal cerebral I/R injury, mice were treated with CpG-ODN (10 μg/25 g body weight) or control-ODN (n=8, 10 μg/25 g body weight) by intraperitoneal injection (i.p.) 1 hour prior to cerebral ischemia (60 minutes) followed by reperfusion.

To evaluate the effect of TLR9 inhibition on cerebral I/R injury, we chose inhibitory CpG-ODN (iCpG-ODN), which binds C-terminal fragment of TLR9 preventing TLR9 activation.[32] iCpG-ODN (n=8, 100 μg/25 g body weight) was administered to mice by i.p. injection 1 hour prior to focal cerebral I/R. Mice that did not receive any treatment served as untreated controls. The CpG-ODN (CpG-ODN 1826), Control-ODN (control-ODN 1826), and iCpG-ODN (iCpG-ODN 2088) were purchased from InvivoGen and dissolved in sterile endotoxin-free water.[20,23]

To examine the therapeutic effect of CpG-ODN on focal cerebral I/R injury, CpG-ODN was administered to mice

15 minutes after focal cerebral ischemia (n=8) by i.p. injection. Focal cerebral ischemia was continued for an additional 45 minutes followed by reperfusion for 24 hours.

To evaluate the effect of CpG-ODN on cerebral functional recovery and survival, CpG-ODN was administered to mice 1 hour prior to cerebral ischemia (60 minutes) followed by reperfusion for up to 21 days.

To determine the role of PI3K/Akt signaling in CpG-ODN induced protection against cerebral I/R injury, the PI3K inhibitor LY294002 (1 mg/25 g body weight) was given to mice 15 minutes before CpG-ODN administration. The mice were subjected to focal cerebral ischemia (60 minutes) followed by reperfusion (24 hours).

Measurement of infarct volume

The infarct volume was determined as described previously.[9,10,30,31] The infarct volume was measured by one blinded to experimental group. After completion of reperfusion, mice were sacrificed and perfused with ice-cold phosphate buffered saline (PBS) via the ascending aorta. Brains were removed and sectioned coronally into 2-mm-thick slices. The slices were stained with 2% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 minutes followed by fixation with 10% formalin neutral buffer solution (pH 7.4). The infarct areas were traced and quantified with an image-analysis system. Unstained areas (pale color) were defined as ischemic lesions. The areas of infarction and the areas of both hemispheres were calculated for each brain slice. An edema index was calculated by dividing the total volume of the left hemisphere by the total volume of the right hemisphere. The actual infarct volume adjusted for edema was calculated by dividing the infarct volume by the edema index.[9,10,30,31] Infarct volumes are expressed as a percentage of the total brain volume±SEM.

Evaluation of neuronal damage in the hippocampal formation (HF)

Neuronal damage in brain sections was examined by Nissl staining as described previously.[9,10,30,31] Paraffin sections cut in the coronal plane at approximately 1.5 mm behind bregma with a thickness of 7 microns were deparaffinized and then stained with 0.1% cresyl violet for 2 minutes. The sections were evaluated using light microscopy.

Evaluation of neurological score

Neurological score was performed by a blinded investigator using a neurological evaluation instrument described in our previous studies.[9,30] The neurological score was evaluated by one blinded to experimental group. Briefly, the scoring system included 5 principle tasks: spontaneous activity over a 3-minute period (0 to 3), symmetry of movement (0 to 3), open-field path linearity (0 to 3), beam walking on a 3 cm×1 cm beam (0 to 3), and response to vibrissae touch (1 to 3). The scoring system ranged from 0 to 15, in which 15 is a perfect score and 0 is death due to cerebral I/R injury. Sham controls received a score of 15.

In situ apoptosis assay

In situ neuronal cell apoptosis was examined by the TdT-mediated dUTP nick end-labeling (TUNEL) assay (Roche Applied Science) as described previously.[30] Fields of hippocampus were randomly evaluated for the percentage of apoptotic cells using the TUNEL assay. The images were viewed on an EVOS-fl digital inverted fluorescent microscopy (Advanced Microscopy Group). Total cells were counted in each field, and apoptotic cells are presented as the percentage of total cells counted.

Immunohistochemistry fluorescent staining

Fluorescent staining was performed to examine caspase-3 activity and microglia activation after cerebral I/R as described previously.[30] Briefly, brains from each group were harvested and immersion-fixed in 4% buffered parafomaldehyde, embedded in paraffin, cut at 7 μm, and stained with an specific anti-cleaved caspase-3 antibody (Cell Signaling Technology, Inc) or anti-Iba-1 antibody (Santa Cruz Biotechnology, Inc) as described previously.[30] After washing, the tissue sections were incubated with FITC-conjugated anti-rabbit (GeneTex) for 1 hour at 25°C and covered with fluorescence mounting medium (Life Technologies). The images were viewed on an EVOS-fl digital inverted fluorescent microscopy (Advanced Microscopy Group). Fields of cortex were randomly examined using a defined rectangular field area for analysis of microglia activation. Total cells were counted in each field, and Iba-1-positive activated microglia cells are presented as the percentage of total cells counted.

In vitro experiments

BV2 microglial cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and penicillin (Gibco) under 5% CO_2 at 37°C.[30] When BV2 cells reached 70% to 80% confluence, they were stimulated with CpG-ODN (100 nmol/L) or Control-ODN (100 nmol/L), respectively, under normoxic conditions for 15, 30, and 60 minutes. The cells were harvested and cellular proteins were isolated for examination of Akt and glycogen synthase kinase (GSK)-3β phosphorylation by Western Blot.[30,33,34]

In separate experiments, BV2 cells were treated with CpG-ODN (100 nmol/L) for 15, 30, and 60 minutes in the presence and absence of LY294002 (20 μmol/L). BV2 cells were also treated with 20 μmol/L LY294002 for 60 minutes.

The cells were harvested for preparation of cellular proteins, which were used for examination of Akt phosphorylation by western blot. There were 6 replicates in each group.

Western blot

Cellular proteins were prepared from brain tissues and western blots were performed as described previously.[8–10,30,31] Briefly, the cellular proteins were separated by SDS-PAGE and transferred onto Hybond ECL membranes (Amersham Pharmacia). The ECL membranes were incubated with the appropriate antibodies, respectively, (anti-phospho-Akt [Ser473], anti-phospho-GSK-3β [Ser9], anti-Bax, anti-Cleaved Caspase-3 [Cell Signaling Technology], anti-Akt, anti-GSK-3β and anti-Bcl2 [Santa Cruz Biotechnology]) followed by incubation with peroxidase-conjugated secondary Abs (Cell Signlaing Technology). The signals were detected with the ECL system (Amersham Pharmacia). To control for lane loading, the same membrane were probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Biodesign) after being washed with stripping buffer. The signals were quantified by scanning densitometry using a Syngene G: Box Image Analysis System.

Caspase-3/7 activity assay

Caspase-3/7 activity in brain tissue was measured using a Caspase-Glo assay kit (Promega) according to the manufacturer's protocol as described previously.[34]

Statistical Analysis

Data is presented in figures as mean±SEM for experimental groups. Group mean levels were compared with analysis of variance (one-way or multifactorial as dictated by the design structure) and the least significant difference procedure (since the F-test was statistically significant). The log-rank test was used to compare group survival trends (Kaplan-Meier plot in Figure 2B). Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results

CpG-ODN Administration Decreased Focal Cerebral Infarct Volume Following I/R

To examine the role of CpG-ODN in focal cerebral I/R injury, we administered CpG-ODN, control-ODN or iCpG-ODN to mice 1 hour before the mice were subjected to cerebral ischemia (1 hour) and reperfusion (24 hours). Figure 1A shows that CpG-ODN administration significantly reduced infarct volume by 69.7% compared with the untreated I/R group (6.4±1.80% vs 21.0±2.85%). Administration of either control-ODN or iCpG-ODN to mice did not alter I/R-induced cerebral infarct volume. We also evaluated the therapeutic

effect of CpG-ODN on cerebral I/R injury. As shown in Figure 1B, therapeutic administration of CpG-ODN at 15 minutes after ischemia significantly reduced infarct volume by 44.7%, when compared with the untreated I/R group (12.6±2.03% vs 22.8±2.54%). Figure 1C shows that cerebral blood flow was significantly reduced by >80% immediately following occlusion of middle cerebral artery and complete reperfusion was restored after the occlusion was released. There was no significant difference in cerebral blood flow between the CpG-ODN, control-ODN, iCpG-ODN, and the untreated I/R groups.

CpG-ODN Administration Improved Neurological Deficits and Increased Survival Rate Following Focal Cerebral I/R

Neurologic score evaluation is an index for the degree of neurologic deficits associated with ischemic stroke.[9,30] We evaluated the effect of CpG-ODN on neurological deficits following cerebral I/R. Figure 2A shows that the neurological score was significantly decreased in untreated I/R mice following cerebral I/R for up to 4 days. In contrast, the neurological scores in CpG-ODN-treated I/R mice were significantly greater than in the untreated I/R group at all time periods. Administration of either control-ODN or iCpG-ODN did not markedly affect cerebral I/R-induced neurological deficits.

We also evaluated the effect of CpG-ODN administration on survival rate following cerebral I/R. As shown in Figure 2B, in untreated mice, 50% of mice died at 48 hours and 80% died at 96 hours following cerebral I/R. In CpG-ODN-treated mice, however, 80% of mice survived at 96 hours and 50% survived at 21 days after cerebral I/R. There was no significant difference in the survival rate between control-ODN, iCpG-ODN, and untreated I/R group.

CpG-ODN Administration Attenuated Neuronal Damage in the Hippocampal Formation

We evaluated the effect of CpG-ODN administration on neuronal morphology in the hippocampus after cerebral I/R. Nissl staining showed that neuronal damage in the cornu ammonis 1 (CA1) field of the hippocampal formation (HF) is characterized by shrunken cell bodies accompanied by shrunken and pyknotic nuclei in the untreated I/R mice (Figure 3). Similar morphological changes were observed in the dentate gyrus (DG) field. In CpG-ODN treated I/R mice, neuronal damage in the HF was significantly decreased and morphology was preserved. Administration of control-ODN or iCpG-ODN did not alter I/R-induced neuronal morphological changes in the hippocampus (Figure 3).
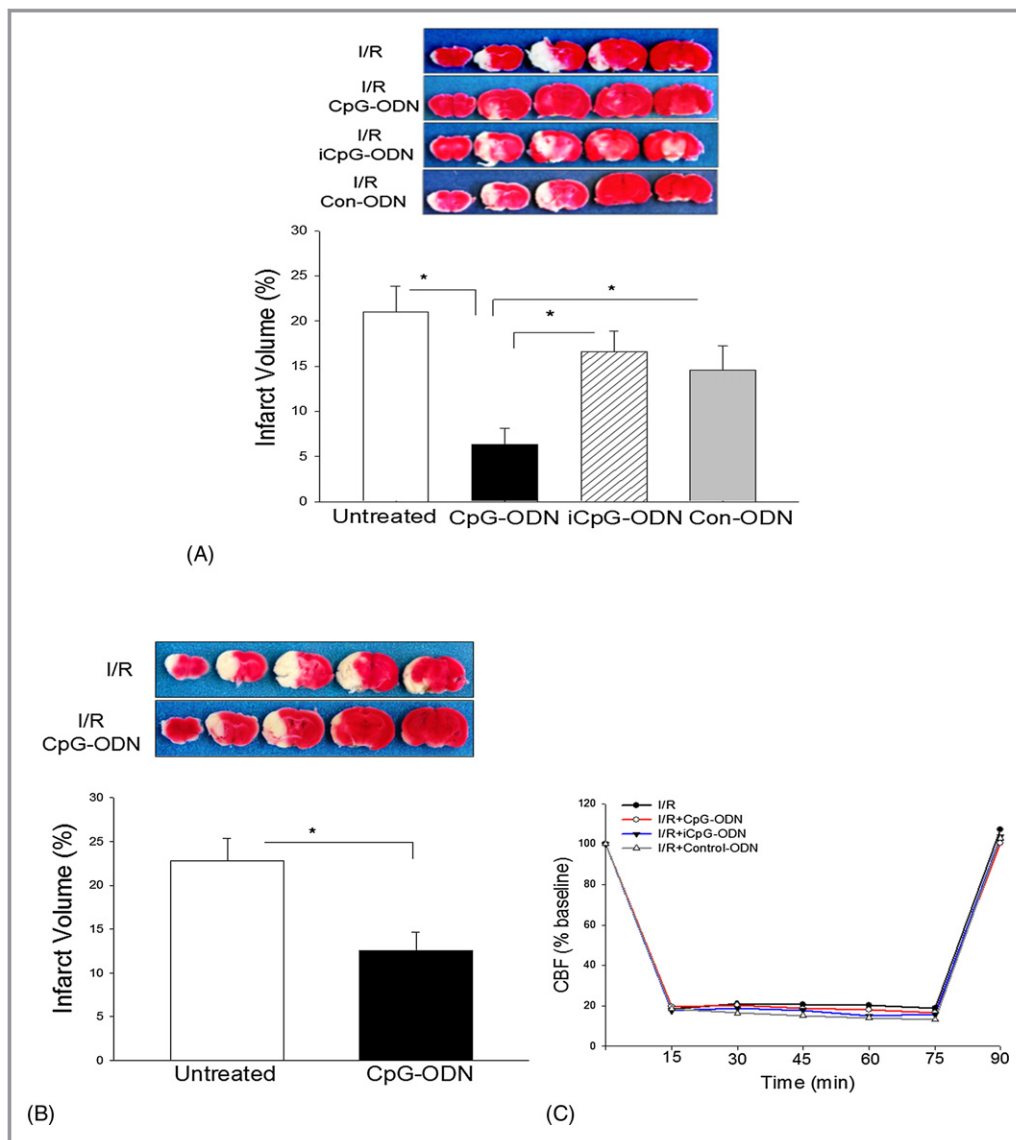
Figure 1. CpG-ODN administration reduces infarct volume following cerebral I/R. A, CpG-ODN (10 µg/ 25 g body weight), control-ODN (10 µg/25 g body weight) or iCpG-ODN (100 µg/25 g body weight) was administered to mice, respectively, by i.p. injection 1 hour prior to cerebral ischemia (n=8/group). B, CpG-ODN (10 µg/25 g body weight) was given to mice 15 minutes after ischemia by intravascular injection. Mice were subjected to cerebral ischemia (60 minutes) followed by reperfusion (24 hours). Representative image of infarct size from groups are shown on the top of the bar graph (n=8 in untreated and n=6 in post treated group). C, Cerebral blood flow measurement before, during and after ischemia (n=6 in untreated, n=6 in CpG-ODN, n=7 in iCpG, and n=6 in control ODN group). *$P<0.05$ compared with indicated group. CBF indicates cerebral blood flow; ODN, oligodeoxynucleotide.

CpG-ODN Administration Attenuated Apoptosis in the Brain Following Cerebral I/R Injury

Cerebral I/R-induced apoptosis plays a role in brain tissue injury in response to I/R.[35] We examined whether administration of CpG-ODN will attenuate cerebral I/R-induced apoptosis in the brain tissues. TUNEL assay showed that cerebral I/R significantly induced apoptosis in the fields of CA1 and cortex (Figure 4A). In CpG-ODN treated I/R mice, however, the numbers of apoptotic nuclei were significantly reduced by 78.8% in CA1 region and by 91.7% in the cortex, respectively, when compared with untreated I/R mice. Cerebral I/R also significantly induced caspase-3 activity as evidenced by showing positive fluorescent staining with a specific anti-cleaved caspase-3 antibody (Figure 4B), when compared with sham control. Caspase-3/7 activity was also
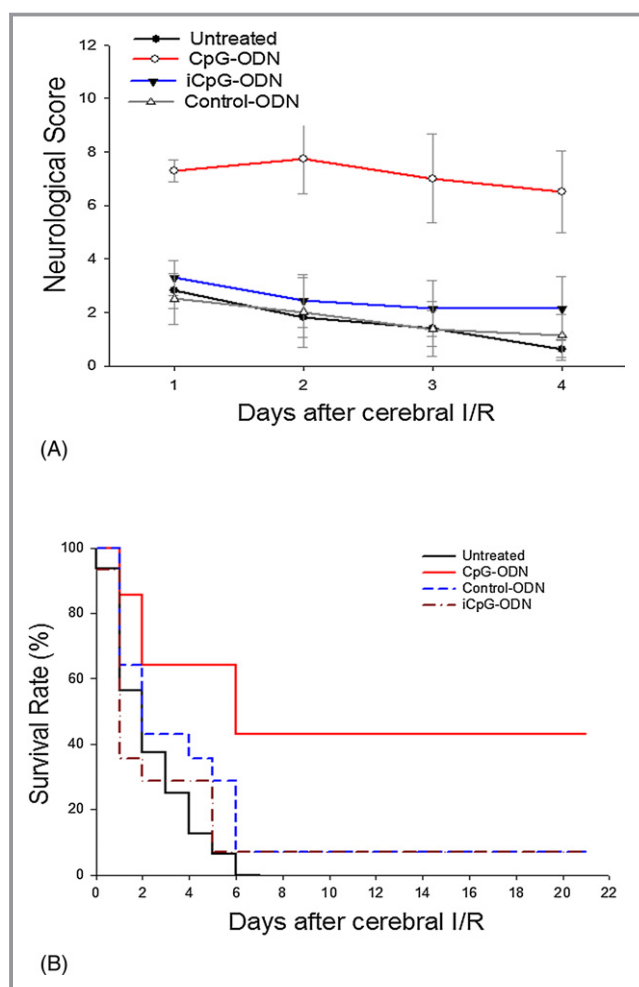
Figure 2. CpG-ODN treatment improved neurological deficits and increased survival rate following cerebral I/R. Mice were treated with CpG-ODN, or control-ODN, or iCpG-ODN, respectively, 1 hour prior to cerebral ischemia (60 minutes) followed by reperfusion for up to 4 to 21 days. Untreated I/R served as I/R control. Sham operation served as sham control. A, Neurological function was evaluated using a scoring system ranging 0 to 15, with 15 being a perfect score and 0 being death due to cerebral I/R injury (n=7 in untreated, n=8 in CpG, n=7 in iCpG, n=7 in control ODN group). B, Survival rate was evaluated following cerebral I/R (n=16 in untreated, n=14 in CpG, n=14 in iCpG, and n=15 in control ODN group). *P<0.05 compared with indicated group. I/R indicates ischemia/reperfusion; ODN, oligodeoxynucleotide.

significantly increased in the brain tissues following cerebral I/R (Figure 4C). In contrast, CpG-ODN administration markedly reduced the numbers of positive fluorescent staining cells and attenuated caspase-3/7 activity, when compared with untreated I/R group. Treatment of mice with control-ODN or iCpG-ODN did not significantly alter cerebral I/R induced apoptosis and caspase-3/7 activity in the brain tissues.

CpG-ODN Administration Increased Bcl-2 and Attenuated Bax Levels in Brain Tissues Following Cerebral I/R

Bcl-2 is important for cell survival and anti-apoptosis while Bax promotes apoptosis.[36] We examined the effect of CpG-ODN administration on the levels of Bcl2 and Bax in the brain tissues following cerebral I/R. As shown in Figure 4D and 4E, cerebral I/R increased the levels of Bax by 126.7% in the brain tissues compared with sham control. In contrast, CpG-ODN administration prevented I/R-increased Bax levels in the brain tissues. CpG-ODN treatment also significantly increased Bcl2 levels by 41% in the brain tissues following cerebral I/R compared with untreated I/R mice (1.24 ± 0.10 vs 0.88 ± 0.14). Administration of either control-ODN or iCpG-ODN to mice did not affect the levels of Bax and Bcl2 in the brain tissues following I/R.

CpG-ODN Administration Attenuated Cerebral I/R-Induced Microglial Cell Activation

Microglia are active sensor and effector cells in the pathophysiological brain injury.[37] We have previously reported that cerebral I/R induced microglia activation in brain tissues.[30] We examined whether CpG-ODN administration will attenuate cerebral I/R-induced microglial cell activation. Figure 5 shows that cerebral I/R induced microglial cell activation as evidenced by anti-Iba-1 positive staining cells in the cortex region of brain (green color). I/R also induced caspase-3 activity (red color) in microglial cells (yellow color in merge). In CpG-ODN treated mice, however, the numbers of anti-Iba-1 positive staining cells were significantly decreased compared with untreated I/R mice ($52.2\pm3.8\%$ vs $74.9\pm3\%$). CpG-ODN administration also markedly reduced caspase-3 activity in microglial cells. Administration of either control-ODN or iCpG-ODN did not markedly alter I/R-induced activation of microglial cells and caspase-3 activity in microglial cells.

CpG-ODN Treatment Increased the Levels of Phospho-Akt and Phospho-GSK-3β in the Brain Tissues

Activation of the PI3K/Akt signaling pathway induces protection against cerebral I/R.[30,38] We examine the effect of CpG-ODN on the activation of PI3K/Akt signaling in the brain tissues. Figure 6A shows that CpG-ODN treatment significantly increased the levels of phospho-Akt in the brain tissues of sham control mice. Cerebral I/R increased the levels of phospho-Akt compared with sham control. However, the levels of phospho-Akt in CpG-ODN treated mice were further
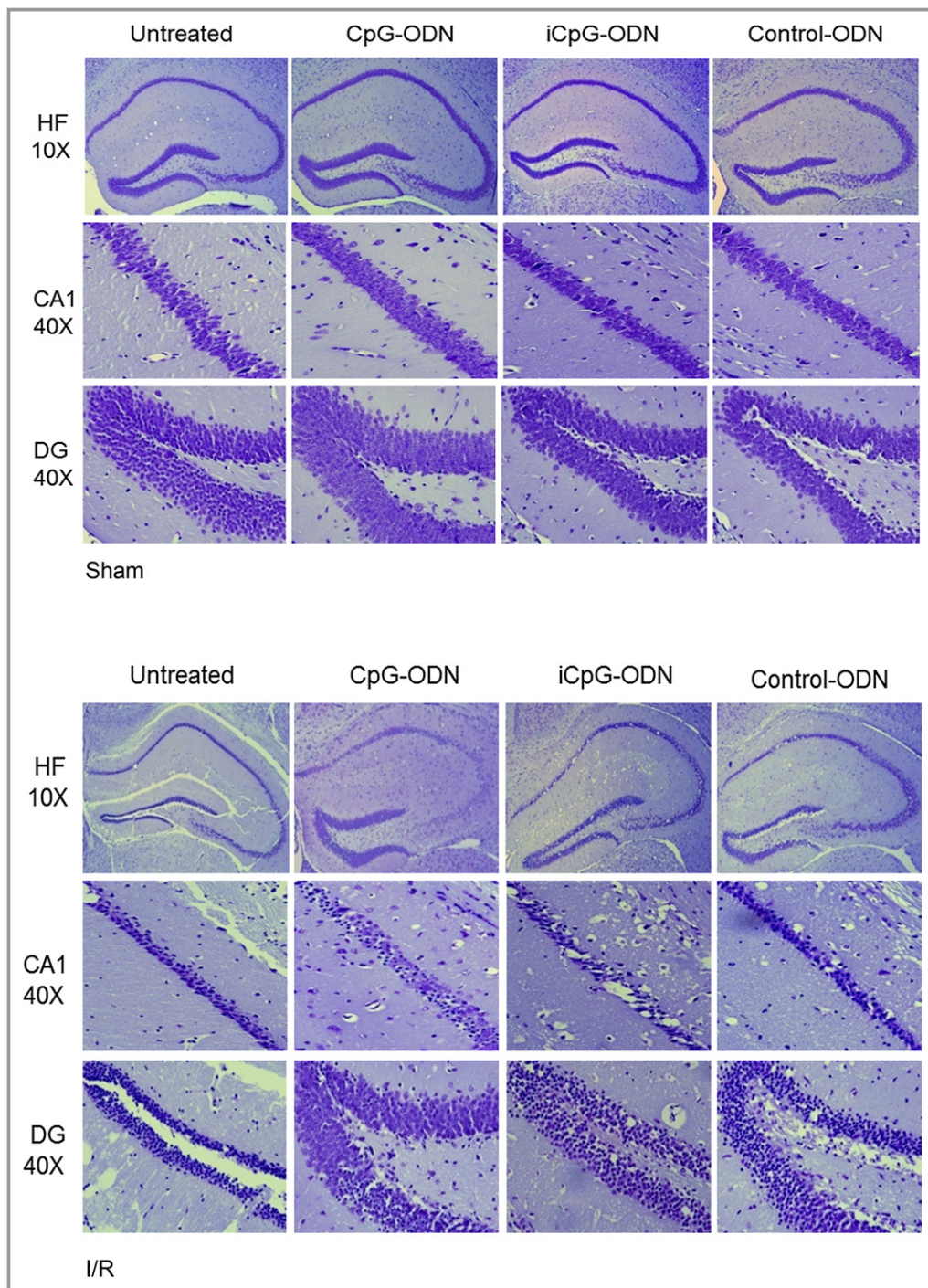
Figure 3. CpG-ODN treatment attenuated neuronal damage in the HF following cerebral I/R. Mice were treated with CpG-ODN, or control-ODN, or iCpG-ODN, respectively, 1 hour prior to cerebral ischemia (60 minutes) followed by reperfusion for 24 hours. Sham operation served as sham control. Brains were harvested, sectioned, and stained with 0.1% cresyl violet (n=4 in each group). HF indicates hippocampal formation; ODN, oligodeoxynucleotide.

increased following cerebral I/R, when compared with untreated I/R group.

GSK-3β is an important downstream target of Akt.[26] Figure 6B shows that CpG-ODN administration markedly increased the levels of phospho-GSK-3β in sham control mice. The levels of phospho-GSK-3β in CpG-ODN treated mice were further increased following cerebral I/R compared with untreated I/R group. Treatment of mice with either control-
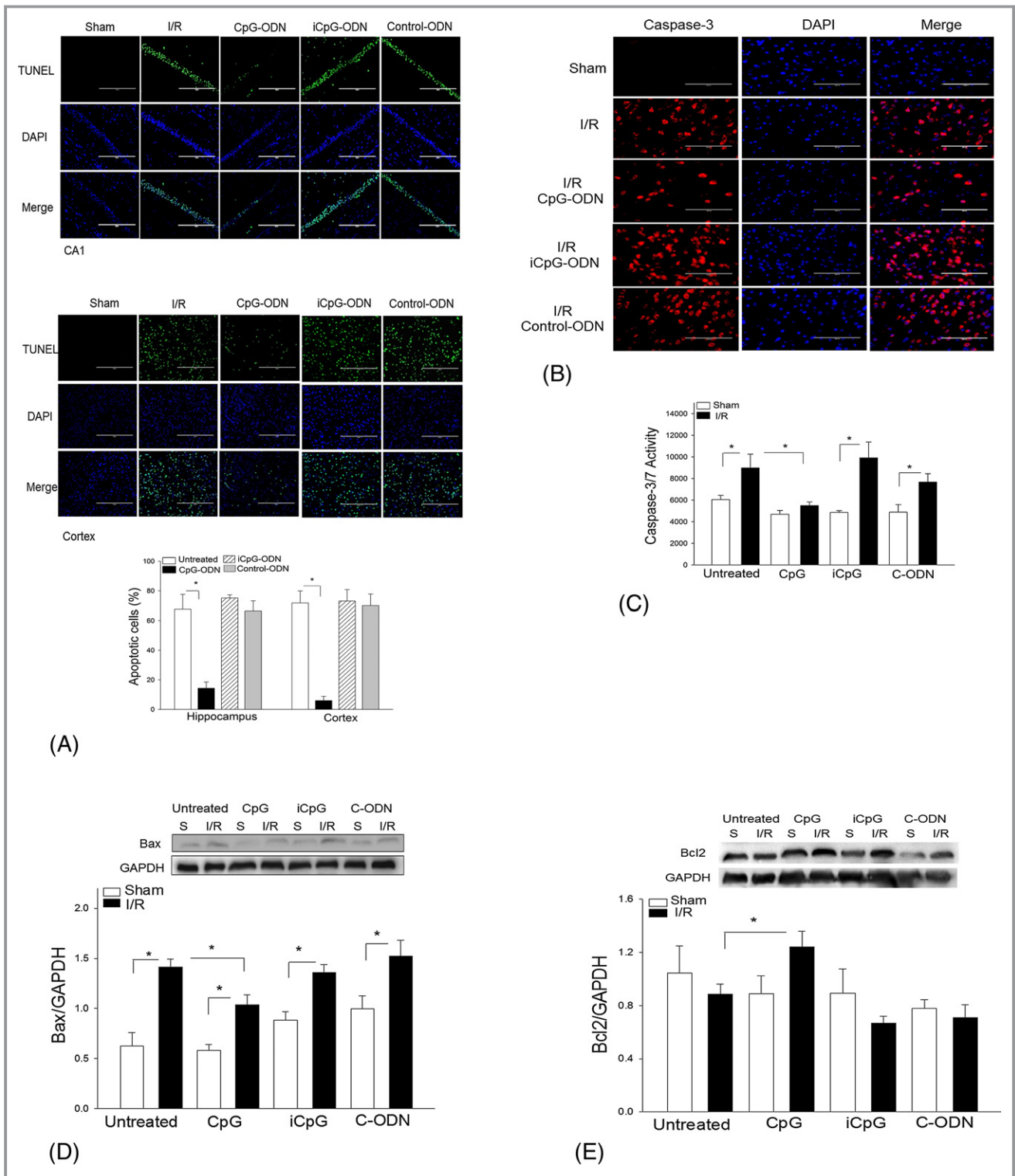
Figure 4. CpG-ODN administration attenuated I/R-induced apoptosis in brain tissues. Mice were treated with CpG-ODN, or control-ODN, or iCpG-ODN, respectively, 1 hour prior to cerebral ischemia (60 minutes) followed by reperfusion for 24 hours (n=6/group). Sham surgical operation served as sham control (n=4). Brains were harvested and sectioned. Cellular proteins were prepared from the remaining brain tissues. A, Apoptosis in brain tissue were examined by TUNEL assay (n=3/group) and (B) caspase-3 activity was stained by cleaved caspase-3 antibody (red). The nuclei were stained by DAPI (blue). C, Caspase-3/7 activity was measured using Caspase-Glo 3/7 assay kit. D and E, CpG-ODN decreased Bax (D) and increased Bcl2 (E) levels in the brain tissues following cerebral I/R. *$P<0.05$ compared with indicated groups. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; I/R indicates ischemia/reperfusion; ODN, oligodeoxynucleotide; TUNEL, TdT-mediated dUTP nick end-labeling.
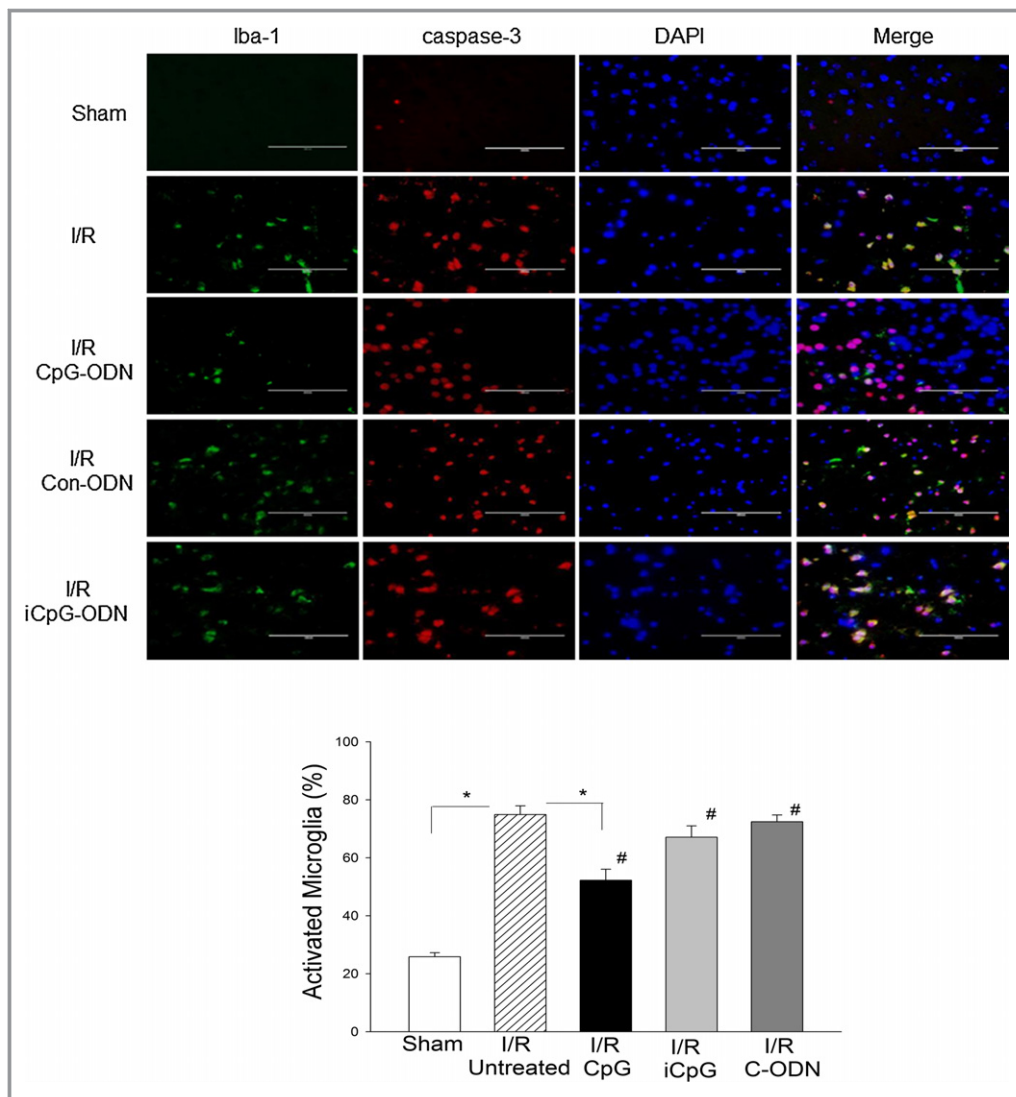
Figure 5. CpG-ODN administration attenuated I/R-induced caspase-3 activity in microglial cells in the brain tissues. Mice were treated with CpG-ODN, or control-ODN, or iCpG-ODN, respectively, 1 hour prior to cerebral ischemia (60 minutes) followed by reperfusion for 24 hours. Sham surgical operation served as sham control. Brains were harvested and sectioned. Activation of microglia was examined with specific antibody against Iba1 (green) and caspase-3 activity was measured by anti-cleaved-caspase-3 antibody (red). Nuclei were stained with DAPI (blue). Caspase-3 activity in activated microglial cells were yellow color (merge). (n=3/group). I/R indicates ischemia/reperfusion; ODN, oligodeoxynucleotide.

ODN or iCpG-ODN did not significantly alter the levels of phospho-Akt and phosopho-GSK-3β in the brain tissues with and without cerebral I/R.

CpG-ODN Induced Akt and GSK Phosphorylation in Cultured Microglial Cells

We performed in vitro experiments using microglial cells (BV2) to investigate whether CpG-ODN can activate PI3K/Akt signaling. BV2 cells were treated with CpG-ODN for 0, 15, 30, and 60 minutes, respectively. Control-ODN served as control. Figures 6C and 6D show that CpG-ODN treatment

significantly induced Akt (C) and GSK-3β (D) phosphorylation at 30 and 60 minutes, compared with the untreated group. PI3K inhibition by LY294002 completely prevented CpG-ODN-induced Akt phosphorylation (Figure 6E). Control-ODN did not markedly stimulate Akt and GSK-3β phosphorylation in microglial cells.

Inhibition of PI3K/Akt Abolished CpG-ODN-Induced Protection Against Cerebral I/R Injury

To determine whether activation of PI3K/Akt signaling contributes to CpG-ODN induced protection against cerebral
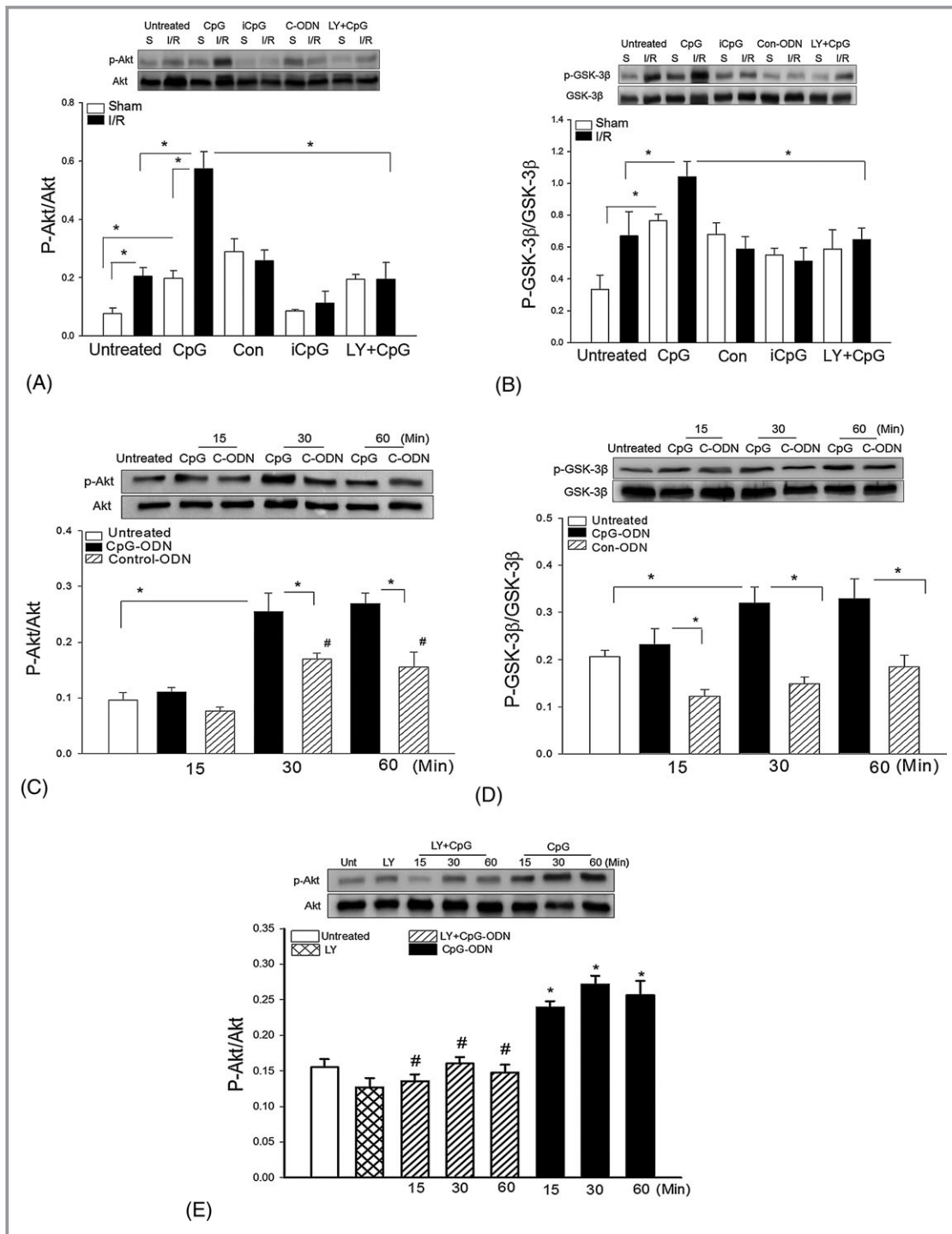
Figure 6. CpG-ODN administration increases levels of Akt and GSK-3β phosphorylation in the brain tissues and in cultured microglial cells. Mice were treated with CpG-ODN (n=7), or control-ODN (n=7), or iCpG-ODN (n=6), respectively, 1 hour prior to cerebral ischemia (60 minutes) followed by reperfusion (24 hours). Untreated I/R mice served as I/R control (n=6). Sham surgical operation served as sham control (n=4). Brains were harvested and cellular proteins were prepared. The phospho-Akt (A) and phospho-GSK-3β (B) were examined by Western blot with specific antibodies. C and D, Microglial cells (BV2) were treated with CpG-ODN or control-ODN for 15, 30, and 60 minutes. The cells were harvested and cellular proteins were prepared Western blot examination of (C) Akt and (D) GSK-3β phosphorylation. E, Microglial cells (BV2) were treated with CpG-ODN for 15, 30, and 60 minutes in the presence and absence of LY294002. PI3K inhibition by LY294002 prevents CpG-ODN-induced Akt phosphorylation in cultured microglial cells (n=6 replicates/group). *P<0.05 compared with indicated groups. GSK indicates glycogen synthase kinase; ODN, oligodeoxynucleotide.
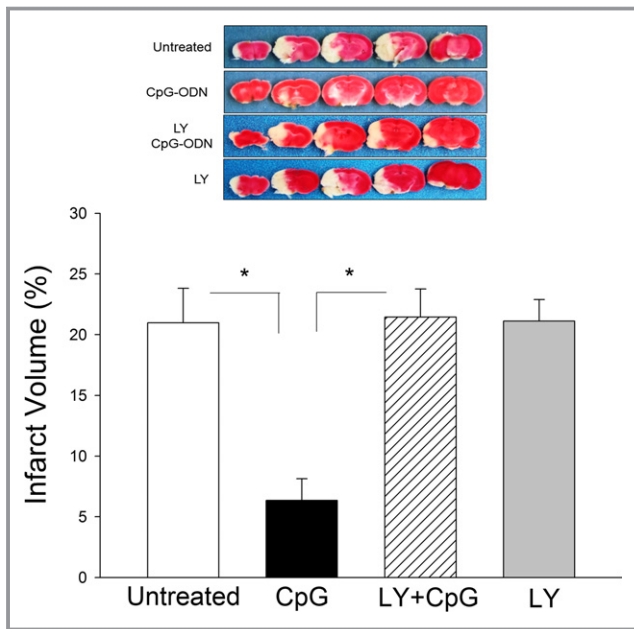
Figure 7. Pharmacologic inhibition of PI3K abrogates CpG-ODN induced protection against cerebral I/R injury. Mice were treated with or without LY294002 (1 mg/25 g body weight) 15 minutes before administration of CpG-ODN. The mice were subjected to cerebral ischemia (60 minutes) followed by reperfusion (24 hours). LY294002 was also administered to mice 15 minutes prior to I/R. Infarct size was examined by TTC staining. n=8/group. *P<0.05 compared with indicated groups. I/R indicates ischemia/reperfusion; ODN, oligodeoxynucleotide.

I/R injury, we treated mice with LY294002, a specific PI3K inhibitor,[20,23,30] 15 minutes prior to CpG-ODN administration. As shown in Figure 7, CpG-ODN administration markedly reduced infarct volume compared with untreated I/R mice. However, the infarct volume in CpG-ODN treated mice was comparable with untreated I/R mice, when LY294002 was administered. Thus, PI3K/Akt inhibition by LY294002 completely abolished protection against cerebral I/R injury by CPG-ODN. There was no significant difference in the infarct volume between CpG-ODN-LY294002 I/R group and untreated I/R mice. Figures 6A and 6B show that LY294002 treatment prevented CpG-ODN-induced increases in the levels of phospho-Akt and phospho-GSK-3β in the brain tissues.

Discussion

The present study has shown that administration of CpG-ODN, a TLR9 ligand, to mice 1 hour prior to cerebral I/R significantly reduced infarct volume, attenuated neurological deflects and improved survival rate following cerebral I/R injury. More significantly, therapeutic administration of CpG-ODN, 15 minutes after ischemia, also markedly decreased cerebral I/R-induced infarct volume. In addition, CpG-ODN administration significantly increased the levels of

phospho-Akt and phospho-GSK3β in the brain tissues. PI3K inhibition abolished CpG-ODN-induced protection against cerebral I/R injury. Thus our data suggest that CpG-ODN attenuates focal cerebral I/R injury via a PI3K/Akt dependent mechanism.

Innate immune and inflammatory responses are known to be involved in cerebral I/R injury.[2–4] TLRs play a critical role in the induction of innate immune responses.[5] TLRs activate signaling through MyD88 and/or TRIF.[5] Recent evidence has indicated that TLRs contribute to cerebral I/R injury.[6,10–12,39] For example, TLR4 deficiency protects the brain from cerebral I/R injury.[6,10–12,39] Hua et al reported that MyD88 or TRIF knockout mice did not show a reduction of cerebral infarction or improvement of neurological deficits following cerebral I/R.[40,41] This observation indicated that the signaling mediated by MyD88 or TRIF may be required for protection. Indeed, we have previously reported that modulation of TLR2 by its ligand, Pam3CSK4 significantly reduced infarct volume and improved neurological score following cerebral I/R injury.[30] Recently Stevens et al reported that activation of TLR9 by its agonist, CpG-ODN, significantly induced protection against ischemic stroke.[14] Hyakkoku et al reported that TLR3 or TLR9 deficiency did not show a neuroprotective effect against cerebral I/R,[42] indicating that TLR3 or TLR9 may serve a protective role in cerebral I/R injury. Indeed, we demonstrated in the present study that administration of CpG-ODN decreased infarct volume and improved neurological score following cerebral I/R. We also observed that therapeutic administration of CpG-ODN decreased infarct volume. Collectively, the data suggest that modulation of TLR9 by its ligand CpG-ODN could be an approach for the treatment and management of ischemic stroke.

Cerebral I/R induces neuronal apoptosis, which contributes to brain injury in response to I/R.[35] It is well known that Bax is a pro-apoptotic molecule whereas Bcl2 is an important anti-apoptotic molecule.[36] We observed that cerebral I/R significantly increased the levels of Bax and caspase-3/7 activity in the brain tissues, which are consistent with the data showing cerebral I/R, induced apoptotic cells in the brain tissues. Importantly, CpG-ODN administration significantly decreased I/R-induced apoptosis and attenuated I/R-increased levels of Bax and caspase-3/7 activity in the brain tissues. In addition, CpG-ODN markedly increased the levels of Bcl2 both in sham control and I/R mice. The data indicate that the anti-apoptotic effect of CpG-ODN[43] may be one of the mechanisms by which CpG-ODN attenuated cerebral I/R injury.

Microglia are active sensors and versatile effector cells in pathological and pathophysiological brain injury.[37] Interestingly, a recent study by Burguillos et al reported that activation of caspase-8 is associated with microglial activation.[44] Activated microglia release substances that cause

neuronal injury.[37,45] We have observed that cerebral I/R induces microglial activation in brain tissues. However, CpG-ODN treatment prevented I/R-induced activation of microglial cells, suggesting that CpG-ODN can prevent microglial activation. Microglial cells are responsible for much of the TLR expression in brain tissue.[45] We have previously reported that TLR2 modulation increased PI3K/Akt signaling in microglial cells.[30] CpG-ODN treatment also increased the levels of Akt and GSK-3β phosphorylation, which may be a possible mechanism for preventing microglial activation by CpG-ODN.

We observed in the present study that CpG-ODN administration significantly induced Akt and GSK-3β phosphorylation both in vivo and in vitro, indicating that CpG-ODN can activate the PI3K/Akt signaling pathway.[20,30] We have previously reported that activation of PI3K/Akt signaling attenuates cerebral I/R-induced brain injury and neuronal apoptosis,[30] protects the myocardium from I/R injury[20] and improves outcome of polymicrobial sepsis.[23] Activation of PI3K/Akt signaling has been reported to protect cells from apoptosis induced by I/R.[27,33,46] Activated PI3K/Akt can inhibit Bax conformational change, thus preventing Bax from translocating and integrating into mitochondrial membranes. PI3K/Akt activation also phosphorylates Bim, leading to dissociation of Bim from Bcl2. In addition, activation of PI3K/Akt may be a negative feedback regulator that prevents excessive innate immune and/or inflammatory responses.[47,48]

We have previously reported that treatment of cardiomyoblasts with CPG-ODN induced TLR9 tyrosine phosphorylation followed by association with the p85 subunit of PI3K,[20,23] resulting in activating PI3K/Akt signaling. We have observed in the present study that CpG-ODN-induced activation of PI3K/Akt signaling both in vivo and in vitro cultured microglial cells, indicating that activation of the PI3K/Akt signaling pathway may contribute to the CpG-ODN-induced protective effect against cerebral I/R injury. To evaluate our hypothesis, we treated mice with a PI3K specific inhibitor, LY294002, before CpG-ODN administration. We observed that PI3K inhibition completely abolished CpG-ODN-induced protection against cerebral I/R injury. The data suggests that CpG-ODN-induced protection against cerebral is mediated via the PI3K/Akt dependent mechanisms.

In summary, administration of CpG-ODN significantly decreased I/R-induced infarct volume and improved neurological score following cerebral I/R. Therapeutic administration of CpG-ODN also markedly reduced I/R-induced infarct volume. CpG-ODN-induced protection against cerebral I/R injury is mediated through activation of PI3K/Akt signaling. The data suggest that the CpG-ODN may be a new approach for the management and treatment of cerebral I/R injury.

Disclosures

None.

References

1. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger V, Rosamond W, Sacco R, Sorlie P, Stafford R, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J; The American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics—2010 update. *Circulation* 2010; 121:e1–e170.

2. Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. *J Neuroimmunol*. 2007;184:53–68.

3. Stoll G. Inflammatory cytokines in the nervous system: multifunctional mediators in autoimmunity and cerebral ischemia. *Rev Neurol*. 2002;158:887–891.

4. del Zoppo GJ. Acute anti-inflammatory approaches to ischemic stroke. *Ann NY Acad Sci*. 2010;1207:143–148.

5. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature*. 1997;388:394–397.

6. Caso J, Pradillo J, Hurtado O, Lorenzo P, Moro M, Lizasoain I. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation*. 2007;115:1599–1608.

7. Tang SC, Arumugam TV, Xu X, Cheng A, Hughal MR, Jo DG, Lathia JD, Siler DA, Chigurapati S, Ouyang X, Magnus T, Camadola S, Mattson MP. Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. *Proc Natl Aad Sci USA*. 2007;104:13798–13803.

8. Hua F, Ma J, Ha T, Xia Y, Kelley J, Williams DL, Kao RL, Browder IW, Schweitzer JB, Kalbfleisch JH, Li C. Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion. *J Neuroimmunol*. 2007;190:101–111.

9. Hua F, Ma J, Ha T, Kelley J, Williams DL, Kao RL, Kalbfleisch JH, Browder IW, Li C. Preconditioning with a TLR2 specific ligand increases resistance to cerebral ischemia/reperfusion injury. *J Neuroimmunol*. 2008;199:75–82.

10. Hua F, Ma J, Ha T, Kelley JL, Kao RL, Schweitzer JB, Kalbfleisch JH, Williams DL, Li C. Differential roles of TLR2 and TLR4 in acute focal cerebral ischemia/reperfusion injury in mice. *Brain Res*. 2009;1262:100–108.

11. Lehnardt S, Lehmann S, Kaul D, Tschimmel K, Hoffmann O, Cho S, Kreuger C, Nitsch R, Meisel A, Weber JR. Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia. *J Neuroimmunol*. 2007;190:28–33.

12. Ziegler G, Harhausen D, Schepers C, Hoffmann O, Rohr C, Prinz V, Konig J, Lehrach H, Nietfeld W, Trendelenburg G. TLR2 has a detrimental role in mouse transient focal cerebral ischemia. *Biochem Biophys Res Commun*. 2007;359:574–579.

13. Marsh B, Stevens SL, Packard AEB, Gopalan B, Hunter B, Leung PY, Harrington CA, Stenzel-Poore MP. Systemic lipopolysaccharide protects the brain from ischemic injury by reprogramming the response of the brain to stroke: a critical role for IRF3. *J Neurosci*. 2009;29:9839–9849.

14. Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule JL, Lessov NS, Simon RP, Stenzel-Poore MP. Toll-like receptor 9: a new target of ischemic preconditioning in the brain. *J Cereb Blood Flow Metab*. 2008;28:1040–1047.

15. Bahjat FR, Williams-Karnesky RL, Kohama SG, West GA, Doyle KP, Spector MD, Hobbs TR, Stenzel-Poore MP. Proof of concept: pharmacological preconditioning with a Toll-like receptor agonist protects against cerebrovascular injury in a primate model of stroke. *J Cereb Blood Flow Metab*. 2011;31:1229–1242.

16. Kumagai Y, Takeuchi O, Akira S. TLR9 as a key receptor for the recognition of DNA. *Adv Drug Deliv Rev*. 2008;60:795–804.

17. Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov*. 2006;5:471–484.

18. Sester DP, Brion K, Trieu A, Goodridge HS, Roberts TL, Dunn J, Hume DA, Stacey KJ, Sweet MJ. CpG DNA activates survival in murine macrophages

through TLR9 and the phosphatidylinositol 3-kinase-akt pathway. *J Immunol*. 2006;177:4473–4480.

19. Mathur S, Walley KR, Boyd JH. The Toll-like receptor 9 ligand CPG-C attenuates acute inflammatory cardiac dysfunction. *Shock*. 2011;36:478–483.

20. Cao Z, Ren D, Ha T, Liu L, Wang X, Kalbfleisch J, Gao X, Kao R, Williams D, Li C. CpG-ODN, the TLR9 agonist, attenuates myocardial ischemia/reperfusion injury: Involving activation of PI3K/Akt signaling. *Biochim Biophys Acta*. 2013;1832:96–104.

21. Rice L, Orlow D, Ceonzo K, Stahl GL, Trianabos AO, Wada H, Aird WC, Buras JA. CpG oligodeoxynucleotide protection in polymicrobial sepsis is dependent on interleukin-17. *J Infect Dis*. 2005;191:1368–1376.

22. Weighardt H, Feterowski C, Veit M, Rump M, Wagner H, Holzmann B. Increased resistance against acute polymicrobial sepsis in mice challenged with immunostimulatory CpG oligodeoxynucleotides is related to an enhanced innate effector cell response. *J Immunol*. 2000;165:4537–4543.

23. Gao M, Ha T, Zhang X, Lam F, Wang X, Kelley JL, Singh K, Kalbfleisch J, Kao R, Williams DL, Li C. The TLR9 ligand, CPG-ODN, attenuates cardiac dysfunction in polymicrobial sepsis via activation of PI3K/AKT and ERK signaling pathways. *Shock*. 2012;37:33.

24. Zhang X, Gao M, Ha T, Kalbfleisch JH, Browder W, Williams DL, Li C, Kao RL. The TLR9 agonist, CpG-ODN 1826, ameliorates cardiac dysfunction after trauma-hemorrhage. *Shock*. 2012;38:146–152.

25. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in medicine: therapeutic applications and developments. *Clin Pharmacol Ther*. 2008;83:761–769.

26. Cantley LC. The phosphoinositide 3-kinase pathway. *Science*. 2002;296: 1655–1657.

27. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation*. 2000;101:660–667.

28. Matsui T, Ling L, del Monte F, Fukui Y, Franke TF, Hajjar RJ, Rosenzweig A. Adenoviral gene transfer of activated phosphatidylinositol 3' -kinase and akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. *Circulation*. 1999;100:2373–2379.

29. Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K, Hallman M. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur J Immunol*. 2003;33: 597–605.

30. Lu C, Liu L, Chen Y, Ha T, Kelley J, Schweitzer J, Kalbfleisch JH, Kao RL, Williams DL, Li C. TLR2 ligand induces protection against cerebral ischemia/reperfusion injury via activation of phosphoinositide 3-kinase/Akt signaling. *J Immunol*. 2011;187:1458–1466.

31. Lu C, Hua F, Liu L, Ha T, Kalbfleisch J, Schweitzer J, Kelley J, Kao R, Williams D, Li C. Scavenger receptor class-A has a central role in cerebral ischemia/reperfusion injury. *J Cereb Blood Flow Metab*. 2010;30:1972–1981.

32. Yang L, Guo W, Zhang Q, Li H, Liu X, Yang Y, Zuo J, Liu W. Crosstalk between Raf/MEK/ERK and PI3K/AKT in suppression of Bax conformational change by Grp75 under glucose deprivation conditions. *J Mol Biol*. 2011;414:654–666.

33. Li C, Ha T, Kelley J, Gao X, Qiu Y, Kao RL, Browder W, Williams DL. Modulating Toll-like receptor mediated signaling by (1−>3)-β-D-glucan rapidly induces cardioprotection. *Cardiovasc Res*. 2003;61:538–547.

34. Ha T, Hu Y, Liu L, Lu C, McMullen JR, Shioi T, Isumo S, Kelley J, Kao RL, Williams DL, Gao X, Li C. TLR2 ligands induce cardioprotection against ischemia/reperfusion injury through a PI3K/Akt-dependent mechanism. *Cardiovasc Res*. 2010;87:694–703.

35. Broughton BRS, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. *Stroke*. 2009;40:e331–e339.

36. Herrmann JL, Beham AW, Sarkiss M, Chiao PJ, Rands MT, Bruckheimer EM, Brisbay S, McDonnell TJ. Bcl-2 suppresses apoptosis resulting from disruption of the NF-kappa B survival pathway. *Exp Cell Res*. 1997;237:101–109.

37. Weinstein JR, Koerner IP, Moller T. Microglia in ischemia brain injury. *Future Neurol*. 2010;5:227–246.

38. Zhao H, Sapolsky RM, Steinberg GK. Phosphoinositide-3-kinase/akt survival signal pathways are implicated in neuronal survival after stroke. *Mol Neurobiol*. 2006;34:249–270.

39. Cao CX, Yang QW, Ly FL, Cui J, Fu HB, Wang JZ. Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. *Biochem Biophys Res Comm*. 2006;353:509–514.

40. Hua F, Wang J, Sayeed I, Ishrat T, Atif F, Stein DG. The TRIF-dependent signaling pathway is not required for acute cerebral ischemia/reperfusion injury in mice. *Biochem Biophys Res Commun*. 2009;390:678–683.

41. Yang QW, Li JC, Lu FL, Wen AQ, Xiang J, Zhang LL, Huang ZY, Wang JZ. Upregulated expression of toll-like receptor 4 in monocytes correlates with severity of acute cerebral infarction. *J Cereb Blood Flow Metab*. 2008;28: 1588–1596.

42. Hyakkoku K, Hamanaka J, Tsuruma K, Shimazawa M, Tanaka H, Uematsu S, Akira S, Inagaki N, Nagai H, Hara H. Toll-like receptor 4 (TLR4), but not TLR3 or TLR9, knock-out mice have neuroprotective effects against focal cerebral ischemia. *Neuroscience*. 2010;171:258–267.

43. Lim EJ, Park DW, Jeong TW, Chin BR, Bae YS, Baek SH. TRAIL is involved in CpG ODN-mediated anti-apoptotic signals. *Oncol Rep*. 2012;27:1213–1218.

44. Burguillos MA, Dejerborg T, Kavanagh E, Persson A, Hajji N, Garcia-Quintanilla A, Cano J, Brundin P, Englund E, Venero JL, Joseph B. Caspase signalling controls microglia activation and neurotoxicity. *Nature*. 2011;472:319–324.

45. Olson JK, Miller SD. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J Immunol*. 2004;173: 3916–3924.

46. Zhou H, Li X-M, Meinkoth J, Pittman RN. Akt regulates cell survival and apoptosis at a postmitochondrial level. *J Cell Biol*. 2000;151:483–494.

47. Fukao T, Koyasu S. PI3K and negative regulation of TLR signaling. *Trends Immunol*. 2003;24:358–363.

48. Ha T, Hua F, Liu X, Ma J, McMullen JR, Shioi T, Izumo S, Kelley J, Gao X, Browder W, Williams DL, Kao RL, Li C. Lipopolysaccharide-induced myocardial protection against ischemia/reperfusion injury is mediated through a PI3K/Akt-dependent mechanism. *Cardiovasc Res*. 2008;78:546–553.