TLR2 Ligand Induces Protection against Cerebral Ischemia/Reperfusion Injury via Activation of Phosphoinositide 3-Kinase/Akt Signaling

Chen Lu, Li Liu, Yuling Chen, Tuanzhu Ha, Jim Kelley, John Schweitzer, John H. Kalbfleisch, Race L. Kao, David L. Williams and Chuanfu Li

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This study examined the effect of TLR2 activation by its specific ligand, Pam3CSK4, on cerebral ischemia/reperfusion (I/R) injury. Mice (n = 8/group) were treated with Pam3CSK4 1 h before cerebral ischemia (60 min), followed by reperfusion (24 h). Pam3CSK4 was also given to the mice (n = 8) 30 min after ischemia. Infarct size was determined by triphenyltetrazolium chloride staining. The morphology of neurons in brain sections was examined by Nissl staining. Pam3CSK4 administration significantly reduced infarct size by 55.9% (p < 0.01) compared with untreated I/R mice. Therapeutic treatment with Pam3CSK4 also significantly reduced infarct size by 55.8%. Morphologic examination showed that there was less neuronal damage in the hippocampus of Pam3CSK4-treated mice compared with untreated cerebral I/R mice. Pam3CSK4 treatment increased the levels of Hsp27, Hsp70, and Bcl2, and decreased Bax levels and NF-κB-binding activity in the brain tissues. Administration of Pam3CSK4 significantly increased the levels of phospho-Akt/Akt and phospho-GSK3-β/GSK-3β compared with untreated I/R mice. More significantly, either TLR2 deficiency or PI3K inhibition with LY29004 abolished the protection by Pam3CSK4. These data demonstrate that activation of TLR2 by its ligand prevents focal cerebral ischemic damage through a TLR2/PI3K/Akt-dependent mechanism. Of greater significance, these data indicate that therapy with a TLR2-specific agonist during cerebral ischemia is effective in reducing injury. The Journal of Immunology, 2011, 187: 1458–1466.
deficiency or by PI3K inhibition. The data suggest that activation of TLR2 prevents focal cerebral ischemic damage, in part, through a TLR2/PI3K-dependent mechanism.

Materials and Methods

Animals
Age- and weight-matched male C57BL/6 mice and TLR2 knockout (KO) mice (B6.129-TLR2tm1kir/J) were obtained from The Jackson Laboratory (Indianapolis, IN). The TLR2 KO mice were backcrossed with C57BL/6 for nine interbreeding generations. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University. The experiments described in this article conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996). The animal care and experimental protocols were approved by the East Tennessee State University Committee on Animal Care.

Focal cerebral I/R
Focal cerebral I/R was induced by occlusion of the middle cerebral artery on the left side as described in our previous studies (5, 6, 23). In brief, mice (23–25 g body weight) were anesthetized by 5.0% isoflurane, and anesthesia was maintained by inhalation of 1.5–2% isoflurane driven by 100% oxygen flow. Mice were intubated and ventilated with room air using a rodent ventilator at a rate of 110 breaths/min with a total delivered volume of 0.5 ml. Body temperature was regulated at 37.0˚C by surface water heating. After the skin incision, the left common carotid artery (CCA), the external carotid artery, 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; Doccoll, Redlands, CA) 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 min, the filament and the CCA clamp were gently removed (reperfusion starts). The collar suture at the base of the external carotid artery stump was tightened. The skin was closed, anesthesia discontinued, and the animal allowed to recover in 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, Stockholm, Sweden). 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 of bregma. A laser–Doppler probe was connected to the probe holder, and regional cerebral blood flow (CBF) was monitored and recorded. The data were 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 determine infarct volume, we subjected mice to focal cerebral ischemia for 60 min, followed by reperfusion for 24 h. The brains were harvested and stained with triphenyltetrazolium chloride (TTC) (5, 6, 23).

To evaluate the role of a TLR2 ligand in focal cerebral I/R injury, we dissolved Pam3CSK4 (catalog no. tlr-psm; InvivoGen, San Diego, CA) in sterile water and injected 0.2 mg/kg body weight (n = 8) 1 h before cerebral ischemia (60 min), followed by reperfusion for 24 h.

To examine the therapeutic effect of a TLR2 ligand on focal cerebral I/R injury, we administered Pam3CSK4 (i.v., 2 mg/kg body weight; n = 8) to the mice 30 min after the beginning of cerebral ischemia. Focal cerebral ischemia was continued for an additional 30 min, followed by reperfusion for 24 h.

To examine the role of TLR2 in Pam3CSK4-induced protection, we treated TLR2 KO mice (n = 8/group) with or without Pam3CSK4 (2 mg/kg body weight) 1 h before the mice were subjected to focal cerebral ischemia (60 min), followed by reperfusion (24 h). The infarct size was determined by TTC staining (5, 6, 23).

To determine whether activation of the PI3K/Akt signaling pathway was involved in TLR2 ligand-induced protection, we treated mice (n = 8) with the PI3K inhibitor Ly294002 (1 mg/25 g body weight) 15 min before administration of Pam3CSK4. The mice were subjected to focal cerebral ischemia (60 min), followed by reperfusion (24 h). The brains were harvested and infarct volume was determined (5, 6, 23).

Measurement of infarct volume
The infarct volume was determined as described previously (5, 6, 23). Twenty-four hours after I/R, mice were sacrificed and perfused with ice-cold PBS via the ascending aorta. Brains were removed and sectioned coronally into 2-mm-thick slices. The slices were stained with 2% TTC solution at 37˚C for 15 min followed by fixation with 10% formalin in 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 (5, 6, 23). Infarct volumes are expressed as a percentage of the total brain volume ± SEM.

Evaluation of neuronal damage in the hippocampal formation
Neuronal damage in brain sections were determined by Nissl’s method as described previously (5, 6, 23). Paraffin sections cut in the coronal plane at ~1.5 mm behind bregma with a thickness of 7 µm were deparaffinized and then stained with 0.1% cresyl violet for 2 min. The sections were evaluated using light microscopy.

Evaluation of neurologic score
Neurologic score was performed by a blinded investigator using a neurologic evaluation instrument described in our previous studies (5). In brief, the scoring system included five principal tasks: spontaneous activity over a 3-min period (0–3), symmetry of movement (0–3), open-field path linearity (0–3), beam walking on a 3 × 1-cm beam (0–3), and response to vibrissae touch (1–3). 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.

Immunohistochemistry double-fluorescent staining
Double-fluorescent staining was performed to examine the response of microglia cells to Pam3CSK4 stimulation in vivo as described previously (4, 5, 15). In brief, brain tissues were immersion fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut at 7 µm, and stained with an Ab directed against phospho-Akt (goat; Cell Signaling Technology, Beverly, MA) at 25˚C for 1 h. After washing, the sections were incubated with Alexa 555-conjugated anti-goat IgG (GeneTex, San Antonio, TX) for 1 h at 25˚C. The sections were washed again before incubation with anti-ionized calcium-binding adapter molecule 1 (Iba1; Santa Cruz Biotechnology, Santa Cruz, CA) at 25˚C for 1 h. After washing, the sections were incubated with FITC-conjugated anti-rabbit (GeneTex) for 1 h at 25˚C. The sections were covered with fluorescence-mounting medium (Vector Labs). The images were viewed on an EVOS-FL digital inverted fluorescence microscope (Advanced Microscopy Group, Bothell, WA).

In vitro experiments
BV2 microglial cells were provided by Dr. Keshvara at Ohio State University and maintained in DMEM supplemented 5% FBS under 5% CO2 at 37˚C. When the cells reached 70–80% confluency, they were treated with Pam3CSK4 at a final concentration of 1 µg/ml for 0, 5, 15, 30, and 60 min with four replicates at each time point. The cells were harvested, and cellular proteins were isolated for examination of Akt and glycogen synthase kinase (GSK)-3β phosphorylation by Western blot. TLR2 tyrosine phosphorylation and association with the p85 subunit of PI3K were examined by immunoprecipitation with specific anti-TLR2 Ab followed by immunoblots with anti-tyrosine and anti-p85 subunit of PI3K, as described previously (15, 24).

Western blots
Cellular proteins were prepared from brain tissues, and Western blots were performed as described previously (4–6, 24). In brief, the cellular proteins were separated by SDS-PAGE and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with the appropriate primary Ab (anti–phospho-Akt [Ser473], anti–phospho–GSK-3β [Ser9], anti–phospho-IkBa [Cell Signaling Technology], anti–GSK-3β, anti-Akt, anti-IkBa, anti-Bcl2, anti-Bax, anti-pTyr20, anti-p85, anti-Hsp27, and anti-Hsp70 [Santa Cruz Biotechnology]), respectively, followed by incubation with peroxidase-conjugated secondary Abs (Cell Signaling Technology). The signals were detected with the ECL system (Amersham Pharmacia). To control for lane loading, we probed the same membranes with anti–GAPDH (Biode-
sign, Saco, ME) after being washed with stripping buffer. The signals were quantified by scanning densitometry using a Bio-Image Analysis System (Bio-Rad).

**Caspase-3 activity assay**

Caspase-3 activity in brain tissue was measured using a Caspase-Glo assay kit (Promega) according to the manufacturer’s protocol, as described previously (15).

**EMSA**

Nuclear proteins were isolated from ischemic cerebral hemispheres, as described previously (4–6, 24). NF-κB–binding activity was examined by EMSA in a 15-μl binding reaction mixture containing 15 μg nuclear proteins and 35 fmol γ-32P–labeled, double-stranded NF-κB consensus oligonucleotide.

**Statistical analysis**

Data are expressed as mean ± SE. Comparisons of data between groups were made using one-way ANOVA, and Tukey’s procedure for multiple range tests was performed. A *p* value <0.05 was considered significant.

**Results**

**Pam3CSK4 administration decreased focal cerebral infarct volume after I/R**

To examine the role of a TLR2 ligand in focal cerebral I/R injury, we administered Pam3CSK4, a specific TLR2 ligand, to mice 1 h before the mice were subjected to cerebral ischemia (60 min), followed by reperfusion (24 h). Fig. 1A shows that Pam3CSK4 administration significantly reduced infarct volume by 55.9% (18.1 ± 2.72 versus 7.98 ± 1.53) compared with the untreated I/R group. We also examined the therapeutic effect of TLR2 ligand on focal cerebral I/R injury. Administration of Pam3CSK4 30 min after the beginning of ischemia significantly reduced infarct volume by 55.8% (18.1 ± 2.72 versus 7.99 ± 1.00) compared with untreated I/R mice. Fig. 1B shows that CBF was significantly reduced by 90% immediately after occlusion of middle cerebral artery and complete reperfusion after the occlusion was released. There was no significant difference in CBF between the untreated cerebral I/R group and the Pam3CSK4-treated group.

**TLR2 deficiency abolished Pam3CSK4-induced protection against cerebral I/R injury**

We examined the role of TLR2 in Pam3CSK4-induced protection against cerebral I/R injury. TLR2 KO mice were treated with or without Pam3CSK4 1 h before cerebral I/R. Infarct size was evaluated. As shown in Fig. 1C, cerebral I/R induced cerebral infarction in TLR2-deficient mice, which was comparable with WT mice. Pam3CSK4-induced protection against cerebral I/R injury was lost in TLR2-deficient mice. The data suggest that TLR2 is essential for mediating the beneficial effect of Pam3CSK4 on cerebral I/R injury.

**Pam3CSK4-attenuated neuronal damage in the hippocampal formation and improved neurologic deficits**

We evaluated the effect of Pam3CSK4 on neuronal damage and neurologic deficits after cerebral I/R. Nissl staining showed neuronal damage in the cornu ammonis 1 field of the hippocampal formation characterized by shrunken cell bodies accompanied by

**FIGURE 1.** Pam3CSK4 administration reduces infarction after cerebral I/R. A, Pam3CSK4 was administered to mice 1 h before (pre-Pam3) or 30 min after ischemia (post-Pam3). Mice were subjected to cerebral ischemia (60 min), followed by reperfusion (24 h). Fig. 1A shows that Pam3CSK4 administration significantly reduced infarct volume by 55.9% (18.1 ± 2.72 versus 7.98 ± 1.53) compared with the untreated I/R group. We also examined the therapeutic effect of TLR2 ligand on focal cerebral I/R injury. Administration of Pam3CSK4 30 min after the beginning of ischemia significantly reduced infarct volume by 55.8% (18.1 ± 2.72 versus 7.99 ± 1.00) compared with untreated I/R mice. Fig. 1B shows that CBF was significantly reduced by 90% immediately after occlusion of middle cerebral artery and complete reperfusion after the occlusion was released. There was no significant difference in CBF between the untreated cerebral I/R group and the Pam3CSK4-treated group.

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shrunken and pyknotic nuclei in the I/R mice for 1 and 3 d (Fig. 2A). Similar changes were variably expressed in the dentate gyrus and cornu ammonis 1. In the Pam3CSK4-treated mice, neuronal damage in the HC was significantly decreased and morphology was preserved.

Neurologic score evaluation is an index for the degree of neurologic deficits associated with stroke. Fig. 2B shows that the neurologic score was significantly decreased in I/R mice after 24 and 72 h of reperfusion. In contrast, the neurologic score of Pam3CSK4-treated I/R mice was significantly higher than that in the untreated

FIGURE 2. Pam3CSK4 treatment attenuates neuronal damage in the hippocampal formation after cerebral I/R. A, Mice ($n = 6/group$) were treated with or without Pam3CSK4 1 h before cerebral I/R. Sham-operated mice ($n = 4/group$) served as sham control. Brains were harvested 1, 2, and 3 d after reperfusion. Brain sections were stained with 0.1% cresyl violet. Original magnification ×10 (left column) and ×40 (middle and right columns). B, Neurologic function was evaluated using a scoring system ranging from 0 to 15, with 15 being a perfect score and 0 being death caused by cerebral I/R injury. C, Pam3CSK4 decreases caspase-3 activity in the brain tissues after cerebral I/R. Mice were treated with or without Pam3CSK4 1 h before cerebral I/R ($n = 6/group$). Sham surgical operation served as sham control ($n = 4/group$). The brains were harvested and cellular proteins were isolated. Caspase-3 activity was measured using a caspase-3 activity kit. *$p < 0.05$ compared with indicated groups.

FIGURE 3. Pam3CSK4 administration decreases I/R-induced increases in NF-κB–binding activity and phosphorylated IκBα levels in the brain tissues. Mice were treated with or without Pam3CSK4 1 h before cerebral ischemia (45 min) and reperfusion (6 h) ($n = 6/group$). Sham surgical operation served as sham control ($n = 4/group$). The brains were harvested, and nuclear and cytoplasmic proteins were isolated. A, NF-κB–binding activity was determined by EMSA. B, Phospho-IκBα levels were examined by Western blot with specific Ab. *$p < 0.05$ compared with indicated groups.
I/R mice at all time periods, indicating that Pam3CSK4 administration attenuated the neurologic deficits associated with stroke.

We also evaluated the effect of Pam3CSK4 treatment on I/R-induced brain apoptosis by measuring caspase-3 activity. Fig. 2C shows that caspase-3 activity was significantly increased by 57.9% in I/R mice compared with sham control. In contrast, Pam3CSK4 treatment prevented I/R-increased caspase-3 activity in the brain tissues.

**Pam3CSK4 administration attenuated I/R-increased NF-κB–binding activity in the brain tissues**

NF-κB activation plays an important role in cerebral I/R injury (25). Fig. 3 shows that I/R significantly increased the levels of NF-κB–binding activity by 55.3% (Fig. 3A) and phospho-IκBα by 62.4% (Fig. 3B) compared with sham control. However, Pam3CSK4 treatment significantly prevented I/R-increased NF-κB binding activity and IκBα phosphorylation in the brain tissues compared with the cerebral I/R group.

**Pam3CSK4 increased Bcl-2 and attenuated Bax levels in brain tissues after cerebral I/R**

Bcl-2 is important for cell survival and antiapoptosis, whereas Bax promotes apoptosis. We examined the effect of Pam3CSK4 treatment on the levels of Bcl-2 and Bax in the brain tissues after cerebral I/R. Fig. 4A shows that Pam3CSK4 administration significantly increased the levels of Bcl-2 by 64.8% in the brain tissues after I/R compared with untreated I/R mice. Fig. 4B shows that cerebral I/R significantly increased the levels of Bax in the brain tissues by 53.2% compared with sham control. In contrast, Pam3CSK4 treatment significantly prevented I/R-increased levels

**FIGURE 4.** Pam3CSK4 administration increases Bcl-2 after cerebral I/R and decreases Bax levels in the brain tissues. Mice were treated with or without Pam3CSK4 1 h before cerebral ischemia (45 min) and reperfusion (6 h; n = 6/group). Sham surgical operation served as sham control (n = 4/group). The brains were harvested and cellular proteins were isolated. Bcl-2 (A) and Bax (B) were examined by Western blot with specific Abs. *p < 0.05 compared with indicated groups.

**FIGURE 5.** Pam3CSK4 administration increases Hsp27 and Hsp70 levels in the brain tissues. Mice were treated with or without Pam3CSK4 1 h before cerebral I/R (6 h; n = 6/group). Sham surgical operation served as sham control (n = 4/group). The brains were harvested and cellular proteins were isolated. Hsp27 (A) and Hsp70 (B) were examined by Western blot with specific Abs. *p < 0.05 compared with indicated groups.
of Bax. The Bax levels in both Pam3CSK4-treated sham control and I/R mice were significantly lower than that of untreated sham and I/R mice (Fig. 4B).

Pam3CSK4 increased the levels of Hsp27 and Hsp70 in the brain tissues after cerebral I/R

Recent evidence has shown that Hsp27 (26, 27) and Hsp70 (28, 29) protect the brain from I/R injury. We examined the effect of Pam3CSK4 administration on the levels of Hsp27 and Hsp70 in the brain tissues after I/R. As shown in Fig. 5A, I/R significantly increased the levels of Hsp27 in the brain tissues by 46.8% compared with sham control. Pam3CSK4 administration also significantly increased the levels of Hsp27 in the brain tissues in sham control by 112% and I/R mice by 43.6% compared with that of untreated sham control and untreated I/R groups. Fig. 5B shows that cerebral I/R significantly decreased the levels of Hsp70 in the brain tissues by 34.9% compared with sham control. In the Pam3CSK4-treated group, the levels of Hsp70 in the sham control and I/R mice were significantly higher than that of untreated sham control and untreated I/R groups.

Pam3CSK4 treatment increased the levels of phospho-Akt and phospho–GSK-3β in brain tissues after cerebral I/R

Activation of the PI3K/Akt signaling pathway protects against cerebral I/R injury (18). Recent evidence demonstrated that stimulation of TLR2 activates the PI3K/Akt signaling pathway (20, 21). We examined the effect of Pam3CSK4 on activation of the PI3K/Akt signaling pathway. Fig. 6A shows that cerebral I/R increased the levels of phospho-Akt in the brain tissues by 140.0% compared with sham control. However, Pam3CSK4 treatment significantly increased the levels of phospho-Akt by 63.5% (Fig. 6A) and phospho–GSK-3β by 95.3% (Fig. 6B) compared with the
untreated I/R group. Microglial cells are active sensors in response to pathogen-associated molecular pattern stimulation (30). We examined whether microglial cells and infiltrated macrophages in brain tissue will respond to Pam3CSK4-increased Akt phosphorylation after cerebral I/R in vivo. Fig. 6C shows that cerebral I/R induced Akt phosphorylation in microglia/macrophages compared with sham control. However, Pam3CSK4 treatment increased Akt phosphorylation in microglia/macrophages in response to I/R, which was consistent with Western blot results (Fig. 6A).

**Pam3CSK4 administration resulted in association between TLR2 and the p85 subunit of PI3K in cultured microglial cells**

Pam3CSK4 treatment increased Akt phosphorylation in microglia/macrophages in brain tissues; therefore, we investigated how Pam3CSK4 treatment will result in Akt and GSK-3β phosphorylation. Microglial cells were treated with Pam3CSK4 (1 μg/ml) for 0, 5, 15, 30, and 60 min, respectively. Figure 6D shows that Pam3CSK4 administration significantly increased the levels of phospho-Akt and phospho-GSK-3β in the cells. LY29004 administration prevented Pam3CSK4-increased Akt and GSK-3β phosphorylation.

Next, we also examined whether Pam3CSK4 treatment will induce TLR2 tyrosine phosphorylation followed by association with the p85 subunit of PI3K. Fig. 6D and 6E show that Pam3CSK4 stimulation increased TLR2 tyrosine phosphorylation and enhanced TLR2 association with the p85 subunit of PI3K. The data suggest that Pam3CSK4 administration activated the PI3K/Akt signaling pathway through TLR2 tyrosine phosphorylation and association with the p85 subunit of PI3K.

**Pharmacologic inhibition of PI3K abrogates the protection against cerebral I/R injury in Pam3CSK4-treated mice**

To examine whether activation of PI3K/Akt signaling will be responsible for protection of the brain from I/R injury in Pam3CSK4-treated mice, we administered the PI3K inhibitor LY294002 to Pam3CSK4-treated mice. Fig. 7 shows that PI3K inhibition with LY294002 abrogated Pam3CSK4-induced protection against cerebral I/R injury. The infarct volume was significantly greater in Pam3CSK4 + LY294002-treated compared with Pam3CSK4-treated mice. There was no significant difference between LY294002 treatment alone and the untreated I/R group.

**Discussion**

A significant finding in this study is that administration of the TLR2 ligand, Pam3CSK4, significantly reduced focal cerebral ischemic injury. Importantly, therapeutic administration of Pam3CSK4, 30 min after cerebral ischemia, also significantly protected against cerebral I/R. However, the beneficial effect of Pam3CSK4 was lost in TLR2-deficient mice, suggesting that TLR2 is required for its ligand-induced protection. In addition, PI3K inhibition abolished Pam3CSK4-induced protection. Thus, our data indicate that administration of a TLR2 agonist attenuates focal cerebral I/R injury via a TLR2- and PI3K/Akt-dependent mechanism.

TLR-mediated signaling pathways are involved in cerebral I/R injury. For example, deficiency of either TLR4 or TLR2 has been shown to protect the brain from cerebral I/R injury (2, 6, 12–14). Recently, Hyakkoku et al. (31) reported that deficiency of TLR3 or TLR9 did not show a neuroprotective effect against cerebral I/R. Hua et al. (32) reported that both MyD88 and TRIF KO mice did not show a reduction of cerebral infarction and improvement of neurologic deficits after cerebral I/R compared with wild type mice (33). It is well known that all TLRs except TLR3 mediate signaling through MyD88 (8, 9), TLR3 and TLR4 mediate signaling through a TRIF-dependent pathway (8, 9). Collectively, current evidence suggests that TLR2 or TLR4 may recognize endogenous ligands (34) and mediate cerebral ischemic injury through yet unidentified mechanisms.

Recent studies have shown that administration of TLR ligands induced protection against ischemic injury through a preconditioning mechanism (5, 17, 35). For example, pretreatment of mice with either a TLR9 ligand (36) or a TLR2 ligand (5) for 24 h significantly protected brain from cerebral ischemic injury. Preconditioning-induced protection requires at least 24 h of pretreatment. We have recently shown that administration of TLR2 ligands, either Pam3CSK4 or peptidoglycan, 1 h before myocardial I/R significantly reduced myocardial infarct size and improved cardiac function (15). However, the myocardial protection by the TLR2 ligands was abolished in TLR2-deficient mice (15), suggesting that TLR2 is required for its ligand-induced protection. In this study, we observed that administration of Pam3CSK4 1 h before cerebral I/R significantly reduced cerebral ischemic injury. More significantly, administration of Pam3CSK4 during the ischemic period also significantly reduced infarct volume after cerebral I/R injury. The data suggest that therapeutic administration of a TLR2 ligand will protect the brain from ischemic insult and may have significant clinical ramifications for the treatment of stroke.

The role of TLR2 deficiency in I/R injury of organs is inconsistent. For example, it has been shown that TLR2 contributes to ischemic injury in liver (37), kidney (38), and heart (39). In contrast, other studies have reported that TLR2 does not contribute to liver I/R injury (40), protects against the small-bowel I/R injury (41), and could be essential for mediating mesenchymal...
stem cell-associated myocardial recovery after acute I/R injury (42). Reports on the role of TLR2 deficiency in cerebral I/R injury are also inconsistent. Ziegler et al. and Lehnardt et al. (13, 14) have reported that TLR2-deficient mice showed decreased infarct volume compared with WT I/R mice, whereas blocking TLR2 by an anti-TLR2 Ab (T2.5) did not reduce infarct volume (43). We have previously shown that TLR2 deficiency did not protect against focal cerebral I/R injury (6). In this study, we also observed that TLR2 deficiency abolished the protection against cerebral I/R injury by Pam3CSK4. It is unclear why there are conflicting observations. The observed differences may be caused by use of different animal models, size of the coated filament used for artery occlusion, animal body weights, different time periods for I/R, and differences in CBF.

Activation of PI3K/Akt-dependent signaling protects the brain from I/R injury and prevents neuronal apoptosis (18, 19). Recent evidence has shown that the PI3K/Akt signaling pathway may be an endogenous negative feedback regulator of TLR signaling (25). We have previously reported that activation of the PI3K/Akt signaling pathway rapidly increases phosphorylation of Akt (15, 24). To investigate whether activation of the PI3K/Akt signaling pathway is involved in TLR2 ligand-induced protection, we examined the levels of phospho-Akt in the brain. We observed that Pam3CSK4 treatment significantly increased the levels of phospho-Akt in the brain tissues after cerebral I/R. Double-fluorescent staining of brain tissues showed that Pam3CSK4 treatment increased Akt phosphorylation in microglia/macrophages (Fig. 6C), suggesting that microglia/macrophages may play a role in the response to Pam3CSK4-induced protection against cerebral I/R injury. Indeed, microglia/macrophages are active sensors and versatile effector cells in pathologic and pathophysiologic brain injury (30), and could be potential therapeutic targets for ischemic brain injury. Microglia/macrophages are responsible for much of the TLR expression in the brain tissue. A recent study by Tang et al. (3) showed that I/R induced high expression of TLR2 and TLR4 in neurons at early time periods, whereas 36 h after I/R, a high expression of TLR2 was observed in microglia. Interestingly, Tang et al. (3) did not observe a response by neurons to the stimulation with TLR2 or TLR4 ligands. We observed in this study that microglia/macrophages responded to the TLR2 ligand Pam3CSK4, stimulation by activating the PI3K/Akt signaling pathway both in vivo and in vitro. Published evidence suggests that there is cross talk between the TLR2 and PI3K/Akt signaling pathways (20, 45). Stimulation of TLR2 resulted in activation of PI3K/Akt-dependent signaling (20, 22). A recent study reported that Mal, an adaptor protein in the TLR-mediated signaling pathway, connects TLR2 to PI3K activation (22). In this study, we observed that treatment of cultured microglial cells with Pam3CSK4 rapidly induced TLR2 tyrosine phosphorylation and increased the association of the p85 subunit of PI3K with TLR2. When considered together, these data suggest that Pam3CSK4 administration increases phosphorylation of TLR2 with subsequent recruitment of the p85 subunit of PI3K, which results in activation of PI3K/Akt-dependent signaling. We speculate that activation of PI3K/Akt signaling in Pam3CSK4-treated mice may be responsible for the protection against cerebral I/R injury. To test this hypothesis, we administered the PI3K inhibitor, LY294002, to Pam3CSK4-treated mice before focal cerebral I/R. We observed that pharmacologic inhibition of PI3K with LY294002 partially abrogated the protective effect of Pam3CSK4 on focal cerebral I/R injury. Thus, we demonstrated that Pam3CSK4-induced protection is mediated, in part, through a TLR2- and PI3K/Akt-dependent mechanism.

The contribution of neuronal apoptosis to cerebral I/R injury has been well documented (46). We observed in this study that Pam3CSK4 administration significantly attenuated caspase-3 activity in the I/R brain tissues, suggesting that TLR2 ligand administration will prevent apoptosis in the ischemic brain. In addition, activation of the PI3K/Akt signaling pathway has been demonstrated to have an antiapoptotic effect (47). Therefore, activation of the PI3K/Akt signaling pathway could be an antiapoptotic mechanism for the TLR2 ligand. To examine whether other antiapoptotic effectors are involved in Pam3CSK4 attenuation of apoptosis after cerebral I/R, we examined the levels of Bcl-2 and Bax in the brain tissues. Bcl-2 is an important antiapoptotic and antinecrotic molecule, whereas Bax is proapoptotic. We observed that Pam3CSK4 treatment significantly increased the levels of Bcl-2 and decreased Bax levels in the brain tissues of both sham control and I/R mice. The data suggest that increased Bcl-2 and decreased Bax levels by Pam3CSK4 in the brain tissues could be another mechanism for the TLR2 ligand protection against cerebral ischemic injury.

Hsp27 has been reported to protect the brain from cerebral I/R injury (26, 27). We have previously reported that increased expression of Hsp27 significantly protects the myocardium from doxorubicin-induced cell death (48). In addition, Hsp70 has been shown to play a protective role in focal cerebral I/R (28, 29). In this study, we observed that Pam3CSK4 treatment increased both Hsp27 and Hsp70 in the brain tissues of sham control and I/R mice. Currently, we do not know how modulation of TLR2 by its ligand, Pam3CSK4, resulted in increased levels of Hsp27 and Hsp70 in brain tissues of both sham and I/R mice. Recent studies have suggested that there is a link between the PI3K/Akt signaling pathway and Hsp27 (35, 49) and Hsp70 (50, 51). For example, we have previously reported that PI3K inhibition attenuated a small dose of LPS-increased Hsp27 expression in the myocardium (35, 49). Because other studies have also reported that inhibition of PI3K resulted in decreased expression of Hsp27 and Hsp70 in brain tissues subjected to cerebral I/R, it is possible that Pam3CSK4 administration increased the levels of Hsp27 and Hsp70 in the I/R brain tissues by activation of the PI3K/Akt signaling pathway.

In summary, either prophylactic or therapeutic administration of a specific TLR2 ligand, Pam3CSK4, to mice significantly reduced cerebral I/R injury. TLR2 deficiency or PI3K inhibition abolished Pam3CSK4-induced protection against cerebral I/R injury, suggesting involvement of a TLR2- and PI3K/Akt-dependent mechanism. In addition, Pam3CSK4 increased the levels of Bcl-2 and decreased the levels of Bax in I/R brain tissues after I/R, which indicates antiapoptosis as an additional mechanism of the Pam3CSK4 protective effects against cerebral I/R injury. The data suggest that therapy using a TLR2-specific agonist could be effective in reducing stroke injury.

Disclosures
The authors have no financial conflicts of interest.

References


