Toll-Like Receptor 4 Is Involved in Subacute Stress–Induced Neuroinflammation and in the Worsening of Experimental Stroke

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Background and Purpose—Psychological stress causes an inflammatory response in the brain and is able to exacerbate brain damage caused by experimental stroke. We previously reported that subacute immobilization stress in mice worsens stroke outcome through mechanisms that involve inflammatory mechanisms, such as accumulation of oxidative/nitrosative mediators and expression of inducible nitric oxide synthase and cyclooxygenase-2 in the brain. Some of these inflammatory mediators could be regulated by innate immunity, the activation of which takes place in the brain and produces an inflammatory response mediated by toll-like receptors (TLRs). Recently, we described the implications of TLR4 in ischemic injury, but the role of TLR4 in stress has not yet been examined. We therefore investigated whether inflammation produced by immobilization stress differs in mice that lack a functional TLR4 signaling pathway.

Methods—We used an experimental paradigm consisting of the exposure of mice to repeated immobilization sessions (1 hour daily for 7 days) before permanent middle cerebral artery occlusion.

Results—We found that TLR4-deficient mice subjected to subacute stress had a better behavioral condition compared with normal mice (C3H/HeN) and that this effect was associated with a minor inflammatory response (cyclooxygenase-2 and inducible nitric oxide synthase expression) and lipid peroxidation (malondialdehyde levels) in brain tissue. Furthermore, previous exposure to stress was followed by a smaller infarct volume after permanent middle cerebral artery occlusion in TLR4-deficient mice than in mice that express TLR4 normally.

Conclusions—Our results indicate that TLR4 is involved in the inflammatory response after subacute stress and its exacerbating effect on stroke. These data implicate the effects of innate immunity on inflammation and damage in the brain after stroke. (*Stroke*. 2008;39:1314-1320.)

Key Words: cerebral ischemia ■ immune system ■ inflammation ■ nitric oxide synthase ■ stroke

A considerable amount of evidence has shown that physical and psychological stress causes inflammation, as shown by the elevation of plasma levels of several proinflammatory cytokines (eg, tumor necrosis factor-α, interleukin-1β) found in animals and humans subjected to psychological stress, anxiety states, and anorexia nervosa.[1–4] It is accepted that this inflammatory response contributes to the cell damage and death found in neurologic and neuropsychiatric diseases, including those related to stress exposure (depression, posttraumatic stress disorder, etc).[5] In the brain, it has been reported that immobilization stress increases inducible nitric oxide synthase (iNOS) and cyclooxigenase-2 (COX-2) activities and expression and produces an accumulation of lipid peroxidation products.[6,7]

These processes could exacerbate brain damage caused by certain pathologies, and in recent years, some studies have shown an exacerbation of stroke outcome in animals subjected to prior social stress.[8,9] We also have demonstrated that prior acute or subacute immobilization stress increases infarct volume and worsens neurologic outcome by actions that include excitotoxicity and inflammatory mechanisms.[10–12]

In this context, innate immunity is a specific immunologic response not only to systemic bacterial infection but also to cerebral injury.[13,14] Activation of this innate immunity takes place in the brain and utilizes Toll-like receptors (TLRs).[13] TLR4, the first-characterized mammalian Toll,[15] is expressed in microglia and astrocytes after inflammation in the central nervous system[16,17] and activates nuclear factor-κB signaling pathways linked to the transcription of many proinflammatory genes that encode cytokines, chemokines, and enzymes such as COX-2 or iNOS, events that have also been demonstrated in inflammation after brain ischemia and psycholog-

ical stress.[18] In addition, mediators that have been isolated after stroke[19] and stress[20] have been identified as ligands of TLR4.

In this context, we recently demonstrated that TLR4 participates in inflammation after stroke, because TLR4-deficient mice had minor infarctions and a lower inflammatory response after middle cerebral artery occlusion (MCAO)[21]; therefore, the aim of this study was to investigate whether innate immunity operative through the TLR4 pathway participates in neuroinflammation and brain damage after immobilization stress.

Materials and Methods

Animals
Adult male C3H/HeN and C3H/HeJ mice weighing 28 to 30 g were used (Jackson Laboratories, Bar Harbor, Me). The C3H/HeJ murine strain does not express functional TLR4 because of naturally occurring mutations in the *TLR4* gene.[22] The C3H/HeN substrain does not express this mutation and was used as the control group. All experimental protocols adhered to the guidelines of the animal welfare committee of the Universidad Complutense (as per DC 86/609/EU). Mice were housed under standard conditions of temperature and humidity and a 12-hour-light/dark cycle (lights on at 8 AM) with free access to food and water.

Experimental Groups
Several groups (n=6 to 9 in each group) were used: (1) a control group (animals were handled for a few seconds once at 11 AM); (2) a subacute stress group (immobilization for 1 hour for 7 days) euthanized 24 hours after the last immobilization exposure (S7); (3) a permanent MCAO group euthanized 24 hours after operation (MCAO); (4) same as group 3 but with prior exposure to stress, operated on 24 hours after the last immobilization exposure and euthanized 24 hours after operation (S7+MCAO).

Immobilization Stress
Mice were restrained, as previously described,[23,24] in 50-mL Corning tubes (11.5 cm long) with modified caps, each with a small hole to accommodate the tail of the mouse. Adequate ventilation was provided by holes drilled into the conical end of the tube and at the sides of the tubes. The tubes did not allow for forward, backward, or rotational movement. Because of the circadian rhythm inherent in corticosterone production, restraint was applied at 10 AM for all experiments. The protocol of stress used was a subacute model consisting of 1 hour of immobilization for 7 days (S7). Animals were humanely killed with an overdose of sodium pentobarbital. After decapitation, the brain was removed from the skull and both cortical areas were excised.

Behavior Characterization
Before euthanasia (immediately after stress in the corresponding groups), animals were tested according to the procedures previously detailed.[25] First, assessment of each animal began with observation of undisturbed behavior in a clear plastic cage: body position (completely flat=0 to upright position=4) and spontaneous activity (none=0 to repeated vigorous movement=3). Then, the animals were transferred to an arena for observation of the following behaviors: transfer arousal (coma=0 to extremely excited=5), gait (absolute incapacity=0 to normal=3), touch escape (none=0 to extremely vigorous=3), and positional passivity (no struggle when held with a hand=0 to maximal struggle=4).

Western Blot Analysis
Samples containing 40 μg protein were loaded, and the proteins were separated by size by 7% to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (at 90 V). Proteins were blotted onto a polyvinylidene difluoride membrane (HybondTM-P, Amersham Biosciences Europe GmbH, Freiburg, Germany) and incubated with specific primary antibodies against iNOS (Santa Cruz, 1:500) and COX-2 (Santa Cruz, 1:1000). Proteins recognized by the antibody were revealed by use of the ECL kit according to the manufacturer's instructions (Amersham Biosciences Europe GmbH, Freiburg, Germany). β-Actin levels were used as loading controls for total cytosolic protein expression.

Lipid Peroxidation
Lipid peroxidation was measured by the thiobarbituric acid test for malonyldialdehyde (MDA) according to the method described by Das and Ratty,[26] with some modifications. The cortex (cerebral tissue, including the infarct core, penumbra, and macroscopically normal surrounding area) was homogenized in 10 volumes of 50 mmol/L phosphate buffer and deproteinized with 40% trichloroacetic acid and 5 mol/L HCl, followed by addition of 2% (wt/vol) thiobarbituric acid in 0.5 mol/L NaOH. The reaction mixture was heated in a water bath at 90°C for 15 minutes and centrifuged at 12 000*g* for 10 minutes. The pink chromogen was measured at 532 nm in a Beckman DU-7500 spectrophotometer.

Induction of Focal Cerebral Ischemia
Mice were anesthetized with 5% isoflurane (in 70% N_2O, 30% O_2) for induction and with 1.5% isoflurane for maintenance. Rectal temperature was maintained at 37°C with use of a heating pad. The MCA was exposed and permanently occluded by electrocoagulation.[27,28] In brief, for MCAO, an incision perpendicular to the line connecting the lateral canthus of the left eye and the external auditory canal was made to expose and retract the temporalis muscle. A burr hole was drilled and the MCA was exposed by cutting and retracting the dura. The MCA was elevated and cauterized. After surgery, the mice were returned to their cages and allowed free access to water and food. The survival rate of the animals until the end of the experiment was 100%.

Infarct Size
Brains were removed 24 hours after MCAO and cut into seven 1-mm coronal brain slices (Brain Matrix, WPI, UK), which were stained in 1% 2,3,5-triphenyltetrazolium chloride (Merck) in 0.1 mol/L phosphate buffer, and infarct size was determined as described.[29] Infarct volumes were measured by sampling stained sections with a digital camera (Nikon Coolpix 990, Nikon Corp, Tokyo, Japan), and the image of each section was analyzed by an image analyzer (Scion Image for Windows 2000, Scion Corp, Frederick, Md). The digitized image was displayed on a video monitor. With the observer masked to the experimental conditions, the contralateral hemisphere perimeter was overlapped onto the ipsilateral hemisphere to exclude edema, and infarct borders were delineated with an operator-controlled cursor. The area of infarct, which was unstained, was determined by counting the pixels contained within the outlined regions of interest and expressed in square millimeters. Infarct volumes (in mm³) were integrated from the infarct areas over the extent of the infarct calculated as an orthogonal projection. All animals displayed infarcts after the occlusion procedure, which included the cortex, subcortex, and striatum, depending on the intensity of the lesion.

Neurologic Characterization
Before euthanasia, neurologic deficits were measured as previously described[30] according to the following graded scoring system: 0=no deficit; 1=flexion of contralateral torso and forelimb on lifting of the whole animal by the tail; 2=circling to the contralateral side, when held by the tail with feet on the floor; 3=spontaneous circling to the contralateral side; and 4=no spontaneous motor activity. Each animal was scored for each of the aforementioned behaviors for ≈1 minute, and the assessment was repeated another 3 times for consistency. The average score for each animal was used.

Table 1. Behavioral Status of TLR4-Deficient Mice (C3H/HeJ) After Immobilization Stress (S7)

Behavior (Points)	Control C3H/HeN	Control C3H/HeJ	S7 C3H/HeN	S7 C3H/HeJ
Body position	4.00	4.00	3.83	4.00
Spontaneous activity	3.00	3.00	2.33	2.67
Transfer arousal	3.14	3.73	3.00	3.17
Gait	3.00	3.00	3.00	3.00
Touch escape	2.83	2.54	2.50	2.83
Positional passivity	4.00	4.00	2.67	3.00
Total score	19.97±0.52	20.27±0.56	17.33±0.2*	18.67±0.3*†

Data are shown as mean score per behavior. See Methods for details. n=6–9 per group.

*$P<0.05$ vs control; †$P<0.05$ vs S7 C3H/HeN.

Assay of MPO Activity

Myeloperoxidase (MPO) activity in brain tissue was determined as an index of neutrophil accumulation, as previously described.[31] In brief, cortex samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide and 0.5% Nonidet P40 (Boehringer) in 20 mmol/L phosphate buffer, pH 6.0, and centrifuged for 10 minutes at 13 000g. Tissue levels of MPO activity were determined on supernatants with H_2O_2 as the substrate for the enzyme. One unit of MPO activity was defined as that converting 1 μmol of H_2O_2 to water in 1 minute at 25°C. Results were expressed in milliunits of MPO per gram wet weight of ischemic tissue.

Plasma Corticosterone Levels

Plasma was obtained from blood samples by centrifuging the sample at 1500g for 10 minutes immediately after stress application. All plasma samples were stored at −80°C before radioimmunoassay of ^{125}I-labeled corticosterone with use of a commercially available kit (DPC, Los Angeles, Calif). A gamma counter (Perkin Elmer Wallac Wizard 1470) was used to measure radioactivity of the samples.

Protein Assay

Proteins were measured with the bicinchoninic acid method.[32]

Source of Materials

All reagents were obtained from Sigma (Madrid, Spain) unless indicated otherwise in the text.

Statistical Analysis

Results are expressed as mean±SEM of the indicated number of experiments; statistical analysis involved 1-way ANOVA, or the Kruskal-Wallis test when the data were not normally distributed, followed by individual comparisons of means (Student-Newman-Keuls test, or Dunn's method when the data were not normally distributed). Comparisons between the groups of mice under 2 different factors (presence or not of stress and gene deficiency) were performed with 2-way ANOVA with a post hoc Newman-Keuls test (intergroup analysis). $P<0.05$ was considered statistically significant.

Results

Behavior Test in TLR4-Deficient Mice After Immobilization Stress

Animals subjected to subacute immobilization stress showed low behavioral scores (a worse functional outcome) when compared with controls. Moreover, TLR4-deficient mice (C3H/HeJ) had higher scores (a better functional outcome) than did the stressed strain with normal expression of TLR4 (Table 1).

Expression of COX-2 and iNOS in TLR4-Deficient Mice After Immobilization Stress

Subacute immobilization stress caused an increase in expression of the inflammatory enzymes COX-2 and iNOS, as we previously demonstrated.[11] However, TLR4-deficient mice (C3H/HeJ) had significantly lower stress-induced expression of both enzymes when compared with control mice (C3H/HeN; Figure 1).

Brain MDA Levels in TLR4-Deficient Mice After Immobilization Stress

Levels of MDA in the brain were determined as an indicator of lipid peroxidation. Subacute immobilization stress caused an increase in both mouse strains, but C3H/HeJ mice had lower levels of MDA than did the strain with normal expression of TLR4 (Figure 2).

Effect of Prior Immobilization Stress on Infarct Volume in TLR4-Deficient Mice After MCAO

Permanent MCAO was performed on C3H/HeJ mice and on C3H/HeN mice, with or without prior exposure to immobilization. TLR4-deficient mice (C3H/HeJ) had a significantly smaller infarct volume (Figure 3A) and infarct areas (Figures 3B and 3C) 24 hours after ischemic injury when compared with control mice (C3H/HeN). When infarct volume was measured 7 days after occlusion, the decrease in infarct volume persisted (data not shown), confirming our previous results.[21] Exposure of mice to 1-hour restraint stress for 7 days increased infarct volume (Figure 3A) and infarct area (Figures 3B and 3C) after permanent MCAO when compared with previously nonstressed animals. Furthermore, restraint stress in TLR4-deficient mice promoted a smaller infarct volume increase than in stressed mice that normally express TLR4 when percentages of worsening (expressed as a percentage of increase in infarcted area) were compared (24% in C3H/HeJ vs 36% in C3H/HeN; $P<0.05$; Figure 3A).

In addition, previous exposure to stress caused a further decrease in behavior and neurologic scores (Table 2) in MCAO animals 24 hours after ischemia, indicating a worse functional outcome in mice with prior stress to experimental stroke. On the other hand, TLR4-deficient mice (C3H/HeJ) showed improved behavior and neurologic outcomes when compared with control mice (C3H/HeN) in both groups with MCAO only and in groups subjected stress before MCAO (Table 2).
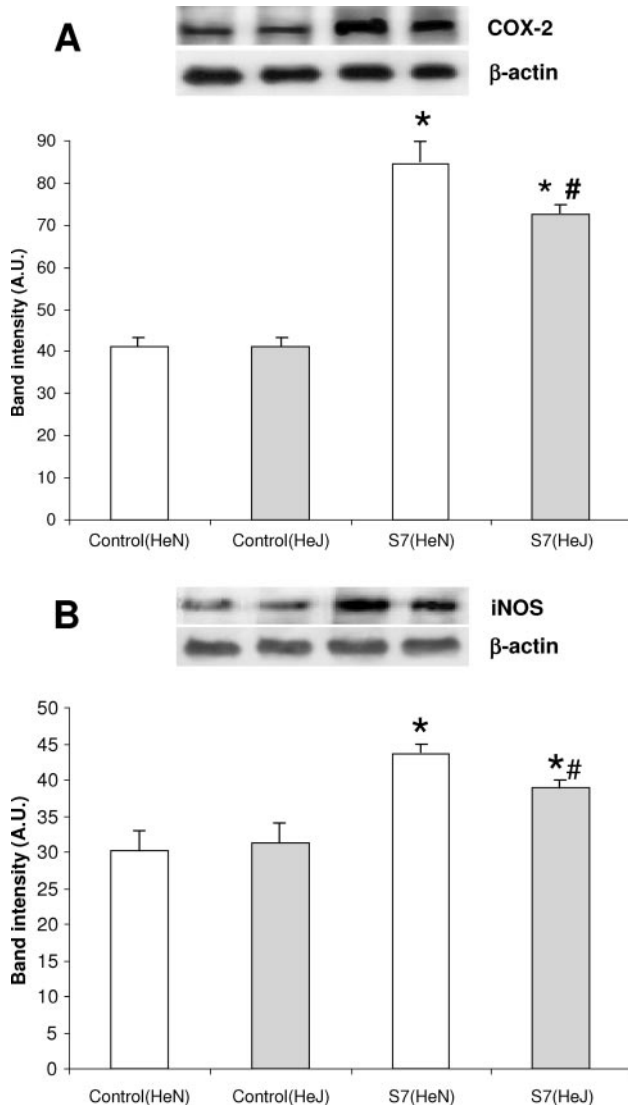
Figure 1. Expressions of COX-2 (A) and iNOS (B) enzymes were analyzed in brains from TLR4-deficient mice [C3H/HeJ (HeJ)] and from mice that express TLR4 normally [C3H/HeN (HeN)] under normal conditions (control) and after prior exposure to immobilization stress (S7). Western blot analysis was performed on brain homogenates, and the corresponding lower panels show the densitometric analysis of bands. Data are mean± SEM, n=6 to 9. *$P<0.05$ vs control; #$P<0.05$ vs S7 (HeN). AU indicates arbitrary units. See Methods for details.

Effect of Prior Immobilization Stress on MPO Activity in TLR4-Deficient Mice After MCAO

Permanent MCAO caused an increase in MPO activity in both mouse strains when compared with their respective controls (MCAO C3H/HeN, 142.7±15.8 mU MPO/g, vs control C3H/HeN, 13.7±3.2 mU MPO/g; MCAO C3H/HeJ, 127.3±9.7 mU MPO/g, vs control C3H/HeJ, 12.4±2.3 mU MPO/g; n=6 to 8, $P<0.05$) as previously described.[31] However, no differences were found in TLR4-deficient mice when compared with mice with normal expression of TLR4. Prior exposure to restraint stress also produced an increase in MPO activity when compared with previously nonstressed animals, and once again, we did not find differences when compared with mice with the TLR4 deficiency (S7+MCAO C3H/HeN,
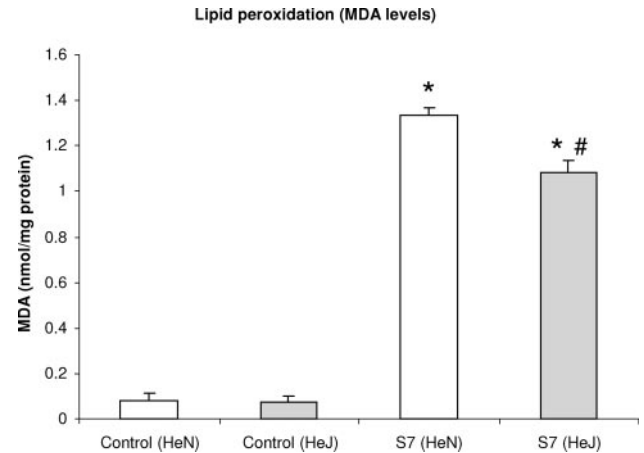
Figure 2. MDA levels were analyzed in brains from TLR4-deficient mice [C3H/HeJ (HeJ)] and from mice that express TLR4 normally [C3H/HeN (HeN)] under normal conditions (control) and after prior exposure to immobilization stress (S7). Data are mean±SEM, n=8. *$P<0.05$ vs control; #$P<0.05$ vs S7 (HeN). See Methods for details.

153.4±5.9 mU MPO/g, and S7+MCAO C3H/HeJ, 139.8± 8.8 mU MPO/g; n=6 to 8, $P>0.05$).

Plasma Corticosterone Levels

Both stress and MCAO procedures increased plasma corticosterone levels, but no differences were found when both mouse strains were compared (Table 3).

Discussion

Our study shows that TLR4-deficient mice subjected to repeated subacute predictable stress (immobilization for 1 hour daily for 7 days) had a better behavior condition compared with mice that express TLR4 normally, and this effect was associated with a decreased inflammatory response (COX-2 and iNOS expression) and lipid peroxidation in brain tissue. Furthermore, restraint stress in TLR4-deficient mice promoted smaller increases in infarct volume after MCAO than in stressed mice that express TLR4 normally. These data demonstrate that immobilization stress at least partly causes neuroinflammation and exacerbates brain damage after stroke by recruiting TLR4 signaling.

First, we found that animals from both strains, subjected to subacute stress, had a worse functional outcome and an increase in the expression of iNOS and COX-2 and lipid peroxidation when compared with previously nonstressed animals. These results are in agreement with our previous data showing that rats exposed to repeated subacute stress showed an increase in the expression of iNOS and lipid peroxidation.[11] Interestingly, we found that TLR4-deficient mice (C3H/HeJ) had a better behavioral condition than did normal mice (C3H/HeN) after this stress protocol, and these data can be linked to the results obtained for the inflammatory parameters. In particular, when we analyzed the expression of the enzymes responsible for inflammatory and oxidative/ nitrosative damage, such as COX-2 and iNOS, we found a lower inflammatory and oxidative/nitrosative response after stress in TLR4-deficient mice (C3H/HeJ) when compared with the inflammatory response in stressed normal mice
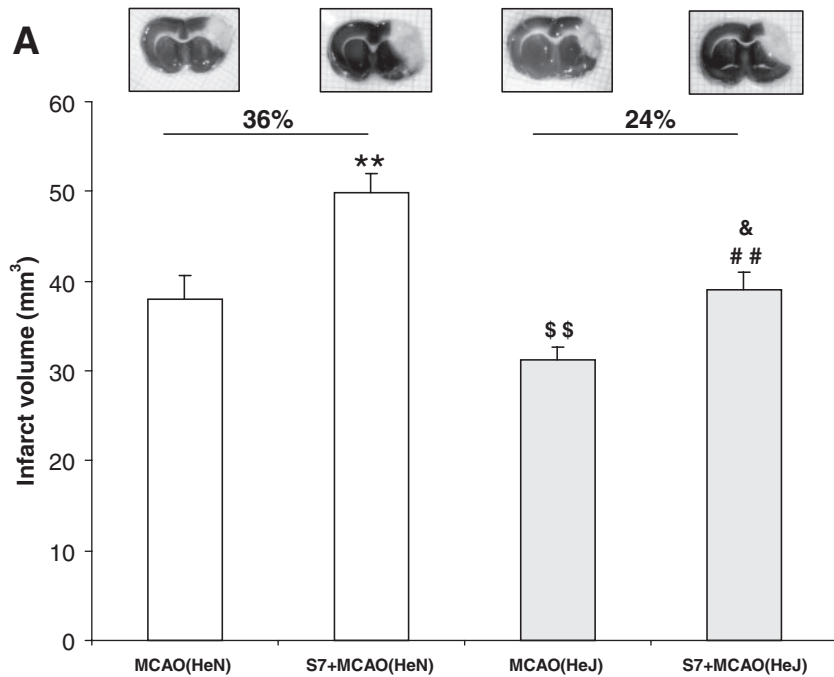
Figure 3. Effect of prior immobilization stress (S7) on infarct outcome in TLR4-deficient mice [C3H/HeJ (HeJ)] after MCAO. Infarct volume (A) and area (B and C) were determined 24 hours after the insult. Data are mean±SEM, n=8. **$P<0.01$ vs MCAO (HeN); ##$P<0.01$ vs MCAO (HeJ); $$$P<0.01$ vs MCAO (HeN); &$P<0.05$ vs S7+MCAO (HeN) (36% vs 24%, $P<0.05$). Photographs are from representative animals.
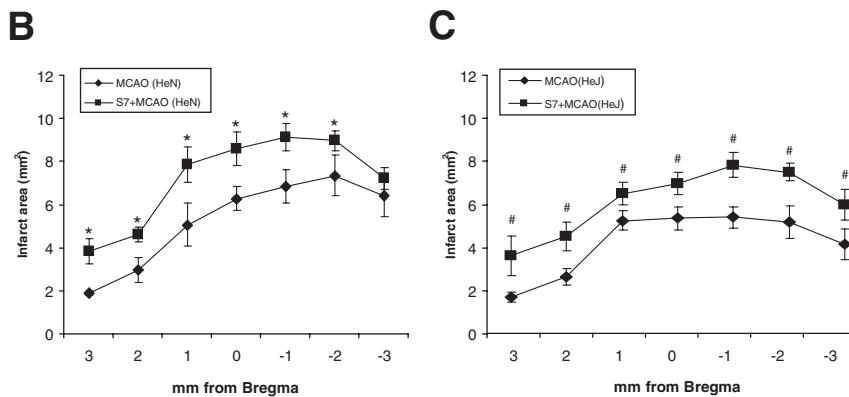
(C3H/HeN). Moreover, when we measured the levels of MDA, a marker of the oxidation of cellular components after repeated restraint stress in the rat brain,[33] we also discovered that TLR4-deficient mice had lower values than did normal mice after a subacute immobilization stress protocol.

To our knowledge, this is the first evidence of the involvement of innate immunity, with respect to the TLR4 receptor, in neuroinflammation caused by stress in the brain. The lower inflammatory response found in TLR4-deficient mice can be explained by the fact that C3H/HeJ mice have a defective response to the ligands of TLR4.[22] It is well known that TLR4 activates nuclear factor-κB signaling pathways that produce the transcription of many proinflammatory genes and enzymes, such as iNOS and COX-2.[15] Therefore, signaling pathways linked to TLR4 in C3H/HeJ mice are likely to be diminished. Furthermore, these results are not due to a different response to stress, because there were no differences in plasma corticosterone levels between stressed C3H/HeN and C3H/HeJ mice.

The present report confirms our previous findings[21] showing that C3H/HeJ mice that lack TLR4 expression have a significantly better outcome after stroke, as shown by a reduction in infarct volume and a substantial recovery of neurologic deficits induced by MCAO compared with C3H/HeN mice that express TLR4 normally. In addition, we demonstrated that restraint stress, which is known to negatively affect experimental stroke outcome, promotes smaller increases in infarct volume in TLR4-deficient mice after permanent MCAO. These data indicate that neuroinflammation caused by stress may exacerbate brain damage induced by ischemia via a mechanism that at least partly involves signaling through the TLR4. The difference in the percentage increase in infarct volume between C3H/HeN and C3H/HeJ mice (36% vs 24%) clearly supports this conclusion, which was corroborated by the improved behavioral and neurologic outcome in TLR4-deficient mice when compared with control C3H/HeN mice.

We also determined postischemic neutrophil infiltration as a component of the neuroinflammatory response. Although immunohistochemistry against MPO is used to evaluate infiltration, we preferred the MPO activity assay because it is a quantitative method. MPO activity was increased 24 hours after ischemic occlusion in both mouse strains, as has been reported.[31] However, we could not find any differences when

Table 2. Effect of Prior Immobilization Stress (S7) on Behavioral and Neurologic Status in TLR4-Deficient Mice (C3H/HeJ) After MCAO

	MCAO C3H/HeN	S7+MCAO C3H/HeN	MCAO C3H/HeJ	S7+MCAO C3H/HeJ
Behavior (points)				
Body position	2.00	2.00	2.00	2.33
Spontaneous activity	2.17	2.17	2.50	2.27
Transfer arousal	2.50	2.17	3.00	2.50
Gait	3.00	2.83	3.00	3.00
Touch escape	2.00	2.00	2.33	2.00
Positional passivity	3.00	2.50	3.00	2.83
Total score	14.67±0.2	13.67±0.3*	15.83±0.3*	14.93±0.2†‡
Neurologic score				
No deficit (0)	0/9	0/6	0/6	0/6
Flexion (1)	2/9	0/6	4/6	0/6
Circling (2)	7/9	3/6	2/6	6/6
Spontaneous circling (3)	0/9	3/6	0/6	0/6
No activity (4)	0/9	0/6	0/6	0/6
Mean score	1.77	2.5*	1.33	2†

Data are shown as mean score per behavior, or as the No. of animals that showed each neurologic score vs the total No. of animals per group. n=6−9 per group. See Methods for details.

*$P<0.05$ vs MCAO C3H/HeN; †$P<0.05$ vs MCAO C3H/HeJ; ‡$P<0.05$ vs S7+MCAO C3H/HeN.

we compared gene deficiency or prior exposure to stress. These data suggest that the TLR4-induced neuroinflammatory response after stroke mainly depends on astrocytic and microglial expression of TLR4, as has been previously demonstrated,[16,17,21] rather than on neutrophil expression of this receptor, although we cannot exclude the possibility of changes at other time points.

As stated previously, TLR4 is expressed in microglia and astrocytes after inflammation[16,17] and after stroke[21]; however, it is not known how stress activates TLR. In this context, several reports in the literature have described endogenous and exogenous ligands that activate TLR4 to produce an inflammatory response,[13,19] including endogenous ligands such as heat-shock proteins 60 and 70,[34,35] which are also induced by stress.[20,36] In addition, we recently showed that stress induces bacterial translocation.[37] It is plausible that bacterial lipopolysaccharide, a known ligand of TLR4, might reach the brain and contribute to damage through TLR4 activation.

In summary, our results indicate that in the brain, TLR4 is involved in the inflammatory response caused by subacute stress. Furthermore, the data presented here provide evidence that stress can increase brain ischemic damage by TLR4-dependent mechanisms. Our data are the first to show that innate immunity participates in brain damage after stress and stroke and open new lines of research. With the consideration that the immune response occurs early after injury, it would be useful to develop new therapies to inhibit TLR4 signaling through the use of neutralizing antibodies or drugs with antagonist characteristics to produce a neuroprotective effect.

Table 3. Effect of Immobilization Stress (S7) and MCAO Procedures on Plasma Corticosterone Levels in TLR4-Deficient Mice (C3H/HeJ)

Groups	Plasma Corticosterone, ng/mL, Mean±SEM
Control C3H/HeN	20.08±3.95
Control C3H/HeJ	24.19±7.19
S7 C3H/HeN	95.23±16.49*
S7 C3H/HeJ	83.13±13.19*
MCAO C3H/HeN	323.96±11.99*†
MCAO C3H/HeJ	335.27±14.22*†
S7+MCAO C3H/HeN	939.35±23.7*†‡
S7+MCAO C3H/HeJ	938.89±26.8*†‡

n=6−9 per group. See Methods for details.

*$P<0.05$ vs control; †$P<0.05$ vs S7; ‡$P<0.05$ vs MCAO.

Disclosures

None.

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