Neuroinflammation and Both Cytotoxic and Vasogenic Edema Are Reduced in Interleukin-1 Type 1 Receptor-Deficient Mice Conferring Neuroprotection

Jelena Lazovic, PhD; Anirban Basu, PhD; Hsiao-Wen Lin; Raymond P. Rothstein; J. Kyle Krady, PhD; Michael B. Smith, PhD; Steven W. Levison, PhD

Background and Purpose—Interleukin-1 (IL-1) is a proinflammatory cytokine implicated in multiple neurodegenerative diseases, including stroke. However, to date, there is no consensus regarding which receptor(s) mediates the detrimental effects of IL-1. We hypothesized that abrogating IL-1 type 1 receptor (IL-1R1) signaling would reduce edema, chemokine expression, and leukocyte infiltration; lower levels of iNOS; and, consequently, decrease free radical damage after mild hypoxia/ischemia (H/I), thus preserving brain cells.

Methods—IL-1R1 null mice and wild-type mice were subjected to a mild H/I insult. MRI was used to measure the area affected at 30 minutes and 48 hours after H/I. An RNAse protection assay was used to evaluate changes in chemokine mRNA expression. RT-PCR was used to assess inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase mRNA levels. Immunohistochemistry was used to assess leukocyte infiltration. Western blots were used to assess iNOS and glutamate aspartate transporter protein levels.

Results—IL-1R1 null mice had reduced cytotoxic and vasogenic edema. The volume of hyperintense signal on T2-weighted images was reduced on average by 90% at 48 hours after H/I. The induction of multiple chemokine mRNAs was significantly reduced in IL-1R1 null mice compared with wild-type mice at 18 and 72 hours after H/I, which correlated with fewer infiltrating CD3+ leukocytes. Levels of iNOS protein and mRNA (but not glutamate aspartate transporter) were significantly reduced in the IL-1R1 mouse brain.

Conclusions—These findings indicate that abrogating IL-1R1 signaling could protect brain cells subsequent to a mild stroke by reducing edema and immune cell recruitment, as well as by limiting iNOS-mediated free radical damage. (Stroke. 2005;36:2226-2231.)

Key Words: cytokines ■ interleukin-1 ■ inducible nitric oxide synthase ■ inflammation ■ leukocytes ■ microglia ■ stroke

The central nervous system is an immune-privileged organ with limited immune cell surveillance; however, after a potentially damaging insult, resident microglia are activated, chemokine production is induced, and peripheral immune cells are recruited. At the onset of inflammation, proinflammatory cytokines, mainly interleukin (IL)-1β and tumor necrosis factor α, are responsible for the activation of endothelial cells, enabling them to recruit macrophages, neutrophils, and T cells across the blood-brain barrier. Among the 3 isoforms of nitric oxide synthase (NOS), endothelial NOS (eNOS) and neuronal NOS are constitutively expressed, whereas inducible NOS (iNOS) is transcribed in response to immunological stimuli. The NO produced by eNOS is synthesized within endothelial cells and is proposed to be protective because of a beneficial vasodilatory effect on the blood vessels seen after hypoxia/ischemia (H/I). In contrast, NO produced by iNOS and synthesized by activated microglia/macrophages and astrocytes has been proposed to contribute to free radical damage after H/I.

Two types of IL-1 receptors have been identified to date: IL-1 type 1 receptor (IL-1R1), which is known to transduce intracellular signals, and the IL-1 type II receptor, which does not directly signal. In earlier work we established that there...
is reduced neuroinflammation in IL-1R1 null mice after a traumatic brain injury. The purpose of this study was to test the hypothesis that there would be reduced edema, chemokine production, leukocyte infiltration, and iNOS in an experimental model of stroke and, consequently, neuroprotection.

Methods

Animals
Adult IL-1R1 (−/−) male mice (3 to 6 months old) that had been backcrossed 9 times onto a C57BL/6 background were interbred to generate the IL-1R1 null mice that were used in these studies. Age-matched C57BL/6 mice were bred to generate wild-type (WT) mice. Animals were maintained at the Hershey Medical Center by the Department of Comparative Medicine, an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility. The experiments were in accordance with research guidelines set forth by the Society for Neuroscience. PCR analysis of tail DNA was used to identify mice carrying the mutant allele.

Induction of Unilateral Cerebral H/I
H/I was induced in adult mice by a modification of the Levine procedure as described previously. Briefly, animals were anesthetized with isoflurane (4% induction and 1.5% maintenance in room air), and the right common carotid artery was double ligated with 3–0 surgical silk. To induce hypoxia, each animal was placed in a 500-ml glass jar partially submerged in a temperature-controlled water bath at 35.5°C and exposed to 8% O2/balance N2 for 22 minutes. During the 22 minutes of hypoxia, 2 of 9 WT animals died, whereas in 2 of 7 WT mice, the cytotoxic edema could impact the evolution of the initial cytotoxic edema into vasogenic edema, the same animals were imaged at 48 hours after H/I.

MRI and Infarct Volume Measurements
MRI (3.0 T, Bruker Instruments) was performed at 30 min (diffusion-weighted sequence) and 48 hours (rapid acquisition with relaxation enhancement (RARE) and spin echo sequence) after H/I. At 30 min after H/I, the mice were simultaneously imaged under isoflurane anesthesia (2%) using a hexagonal coil. A diffusion-weighted imaging sequence was used with 4 slices, 1-mm thick (0.5-mm slice separation), repetition time (TR)/echo time (TE) = 1500/68 ms, Δ = 35 ms, δ = 20 ms, b-value = 730, 625×625 μm resolution, and 1 average, 3 minutes 12 s total imaging time. At 48 hours, mice were anesthetized with 2 mg/kg of xylazine, 15 mg/kg of ketamine, IP and imaged using a T2-weighted RARE sequence (10 slices, 0.5-mm thick slices, TR/TE = 3000/69.4 ms, 78×78 μm resolution, and 8 averages). To quantify T2 weighted changes, each mouse was imaged with a T2-weighted multi echo spin echo sequence. The T2-weighted spin echo sequence had TE = 10 to 152 ms, 15 echoes, TR = 3000 ms, 156×78 μm resolution, and 2 averages, with the same slice thickness and position as the RARE images. Images (RARE scans) were segmented by setting an average, with the same slice thickness and position as the RARE images. Images (RARE scans) were segmented by setting an area in each slice and corrected for brain edema as described previously. Infarct volume was calculated based on the damaged area in each slice and corrected for brain edema as described previously. Infarct volume was calculated based on the damaged area in each slice and corrected for brain edema as described previously. T2 maps were calculated on a pixel-by-pixel basis from the multi echo images.

RNase Protection Assay and RT-PCR
Six WT and 6 IL-1R1 null mice were euthanized at 24 or 72 days after H/I and perfused with medium containing 7 U/mL heparin and then with diethyl pyrocarbonate-treated PBS. RNA was isolated from the neocortex of each hemisphere, and the Pharmingen mCK-5b multiprobe RNase protection assay kit was used to quantify levels of the chemokines macrophage inflammatory peptide-1 (MIP-1) α and β, regulated on activation normally T-expressed and presumably secreted (RANTES), macrophage chemotactic peptide (MCP), and lymphotactin (Ltn).

For RT-PCR, total cellular RNA was reverse transcribed using oligo-dT and random nonamers. RT-PCR was performed according to an earlier published method and using iNOS, eNOS, and cyclophilin primers (Table 1).

Immunohistochemistry
Six WT C57BL/6 and 7 IL-1R1 knockout mice were subjected to H/I and perfused at 72 hours after H/I with 3% formaldehyde, paraffin embedded, and sliced at 6-μm sections. Antigen retrieval was performed using citrate buffer followed by heating in a pressure cooker. Sections were incubated for 2 hours at 37°C with rabbit anti-human CD3 (DAKO). The number of CD3-positive cells per section was scored in the damaged hemisphere. The section was selected to include the infarct and penumbra (or the matching area if the animal was uninjured), and a score between 0 and 4 was assigned by a blinded observer, with 0: no cells; 1: 1 to 10 cells; 2: 11 to 25 cells; 3: 26 to 50 cells; and 4: >50 cells per section.

Western Blotting
Western blotting for glutamate aspartate transporter (GLAST) and iNOS was performed at 48 hours after H/I on 10 μg protein using rabbit anti-GLAST, 1:1000 (Alpha Diagnostic International), and rabbit anti-iNOS (Chemicon International) using chemiluminescence detection. Blots were reprobed with anti-β-tubulin (Santa Cruz Biotechnology).

Statistical Analysis
Normally distributed data sets were analyzed by ANOVA with Fisher LSD post-hoc test, where P < 0.05 was considered statistically significant. Kruskal-Wallis 1-way ANOVA on ranks was performed with a Student-Neuman-Keuls post hoc test on samples that were not normally distributed. For the damage volume comparison between WT and IL-1R1 null mice, the Wilcoxon 2-sample test was used. Sigma Stat software (Jandel Scientific, San Rafael, CA) was used for analysis.

Results
During the 22 minutes of hypoxia, 2 of 9 WT animals died, whereas none died among 6 IL-1R1 nulls. To determine whether decreased signaling through the IL-1R1 would influence the early signal intensity changes associated with cytotoxic edema, 7 adult WT mice and 6 mice lacking the IL-1R1 were compared using diffusion-weighted imaging 30 minutes after H/I. To determine whether decreased IL-1R1 signaling could impact the evolution of the initial cytotoxic edema into vasogenic edema, the same animals were imaged at 48 hours after H/I. A hyperintense signal on diffusion-weighted images, representing cytotoxic edema, was observed in 5 of 7 WT mice and in 1 of 6 IL-1R1 null mice at 30 minutes of recovery. In 3 of 7 WT mice, the hyperintense signal on T2-weighted images involved both the neocortex and striatum, whereas in 2 of 7 WT animals, the cytotoxic edema

<table>
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<tr>
<th>Primer</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>iNOS</td>
<td>5′-CCCCTGCCAAATTGCTTGGCAAGCAG-3′</td>
<td>5′-GGCTGTCAAGGCTGTCGGCTTGG-3′</td>
</tr>
<tr>
<td>eNOS</td>
<td>5′-TTCGCTGGCAGGCTGATCTGCAA-3′</td>
<td>5′-AACATATGCTCTTGCAGAGCCA-3′</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>5′-CCATCCGTGTACAAAGGACTTCAT-3′</td>
<td>5′-TGCCATCCAGGAGGAGCT-3′</td>
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TABLE 1. Oligonucleotide Primers Used for RT-PCR
began in the neocortex, but by 48 hours, the insult evolved into vasogenic edema that encompassed both the neocortex and the striatum (Figure 1).

Of 6 IL-1R1 null mice, only 1 exhibited cytotoxic edema that included both the neocortex and the striatum (Figure 1). At 48 hours after H/I, however, the vasogenic edema in this animal was restricted to the striatum. The neocortex had near-normal T2 values (Figure 1). In the animals that did not have any hyperintense signal present on diffusion-weighted images, T2 values were normal at 48 hours after H/I, indicating that the initially observed protection was not followed by delayed injury. The IL-1R1 null mice had, on average, 90% less total T2-hyperintense volumes at 48 hours after H/I ($P<0.05$; Figure 2).

mRNAs for the chemokines MCP-1, MIP-1, MCP, RANTES, and Ltn generally increased 2-fold in the H/I hemisphere of WT animals by 18 hours of recovery, but significantly lower levels were induced in the damaged hemisphere of IL-1R1 null mice compared with their WT counterparts (Figure 3). Significantly less chemokine mRNAs were present in the damaged hemisphere of IL-1R1 null mice compared with the same hemisphere in WT mice ($P<0.05$; $P<0.01$), consistent with significantly reduced ($P<0.01$ by Kruskal-Wallis nonparametric analysis) recruitment of the CD3+ leukocytes (detected immunohistochemically; Figure 3F and Table 2). In the undamaged contralateral hemisphere, levels of these chemokines were lower in the knockout animals as well, suggesting that IL-1 signaling influences the basal expression of these chemokines.

InOS induced after ischemia contributes to the pathogenesis of neuronal ischemic injury. In accord with published studies, levels of iNOS mRNA and protein were significantly induced in the damaged hemispheres of both strains of mice (Figure 4). However, basal levels of iNOS mRNA in the hemisphere contralateral to the ligation were significantly lower in the IL-1R1 null mice than in WT mice at both 18 and 72 hours ($P<0.05$; $P<0.01$). Moreover, levels of iNOS mRNA induced in the damaged hemisphere in the IL-1R1 null mice were not statistically different from the levels observed in the undamaged hemisphere of the WT mice at both 18 and 72 hours (Figure 4). By contrast, there was a trend toward higher levels of eNOS mRNA levels in the damaged hemisphere, but this increase was not significantly different in the 2 mouse genotypes at 18 hours after H/I (Figure 4).

Consistent with increased levels of iNOS mRNA, protein levels of iNOS increased 4-fold at 48 hours in the ipsilateral hemisphere of WT mice ($P<0.01$) compared with the levels in the same hemisphere of IL-1R1 null mice (Figure 5). iNOS levels increased after H/I, but there was no significant difference from levels expressed in the undamaged WT brain. Similar to chemokines expression, in the IL-1R1 null mice the basal levels of iNOS were lower than those of the WT animals.

After cerebral ischemia, a massive increase in excitatory amino acid release has been documented leading to an adaptive increase in the expression of the glutamate transporter GLAST. GLAST protein levels increased in both mouse genotypes ($P<0.01$) in the ipsilateral hemisphere compared with the contralateral hemisphere at 48 hours after H/I (Figure 5).
Discussion

These studies reveal reduced cytotoxic edema and significantly reduced ischemic brain injury 48 hours after mild H/I. Whereas previous reports have demonstrated that mice deficient in IL-1 ligands or in IL-1/NIH3252 converting enzyme are protected from ischemic insult,3,14 the present data reveal that it is the IL-1R1 that conveys the stimulus. Moreover, the data provide several key insights into how activating this receptor causes neocortical neurodegeneration.

Reduced cytotoxic edema in the IL-1R1 null mice suggests preserved Na+/K+ ATPase function. During H/I, ATP levels fall, Na+/K+ ATPase activity is decreased or suspended, and water follows the influx of sodium ions as they move along their concentration gradient into the energy-starved cells resulting in cytotoxic edema. Because studies in vitro indicate that IL-1β can depolarize neurons, the absence of IL-1R1 signaling likely reduces the frequency of neuronal depolarization, thus indirectly reducing energy consumption to confer neuroprotection.15 Additionally, IL-1 signaling, via NO production, can enhance synaptic glutamate release and potentiate N-methyl-D-aspartate receptor excitotoxicity by increasing calcium influx through the N-methyl-D-aspartate receptor. This would be another means through which abrogating IL-1 signaling can reduce cytotoxic edema.16

The expression of chemokines in the damaged hemisphere of the receptor null mice was not significantly different from that seen in the contralateral hemisphere of WT animals. Lower levels of these chemokines in the contralateral hemisphere of IL-1R1 null animals indicate that expression of basal levels of these chemokines depends on IL-1R1 signaling. Decreased expression of chemokines provides a mechanism to explain the decreased leukocyte infiltration and microglial activation observed here and as recently reported by Basu et al.17 Because iNOS is expressed by mononuclear and polymorphonuclear cells, these data explain the decreased level of iNOS in the IL-1R1 null cortex.18

### Table 2. Fewer CD3-Positive Cells Migrate into the Damaged Hemisphere of IL1-R1 Null Mouse Compared With WT After H/I

<table>
<thead>
<tr>
<th>Animal Type</th>
<th>Mean Score</th>
<th>No. of Animals Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1R1 null</td>
<td>1.14*</td>
<td>7</td>
</tr>
<tr>
<td>WT</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
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Fewer CD3-positive cells migrate into the IL1-R1 null mouse brain after H/I vs WT. The no. of CD3-positive cells per section was scored in the damaged hemisphere by a blinded observer, and a score between 0 and 4 was assigned, with 0: no cells; 1: 1–10 cells; 2: 11–25 cells; 3: 26–50 cells; 4: >50 cells per section.

*P*<0.01 by Kruskal-Wallis nonparametric analysis.
phages and activated microglia are likely sources of the persistent iNOS expression obtained after mild H/I, because macrophages/activated microglia accumulate after mild H/I and we did not observe an increase in neutrophils at the 72-hour time point (H.-W. Lin, unpublished observations, 2005). The observed lower chemokine levels in IL-1R1 null mice are the consequence of abolishment of IL-1R1 signaling and not lower iNOS levels, because iNOS−/− null mice had elevated mRNA levels of chemokines (MIP-1α, MIP-1β, and MCP) after middle cerebral artery occlusion.

NO production and glutamate excitotoxicity subsequent to inflammation also have been implicated in the delayed neurodegeneration after ischemia. Increased NO release from activated leukocytes and astrocytes leads to the production of peroxynitrite, which can irreversibly damage lipids and proteins causing cell death, and extensive neocortical injury occurs as a consequence of enhanced glutamate excitotoxicity after IL-1β injection into the striatum or hypothalamus. These are not separate pathophysiological mechanisms, because NO can enhance glutamate excitotoxicity. Although iNOS−/− mice have lower infarct volumes compared with WT, the neuroprotection is only partially observed in the neocortex. Our results using the IL-1R1 null mice, as well as previous reports using IL-1α/IL-1β double knockout mice, indicate greater neuroprotection of the neocortex. The added neuroprotection observed in the IL-1 mutant mice suggests that additional mechanisms beyond reduced iNOS are protecting the brain from damage. For instance, cyclooxygenase-2 levels are abrogated after a stab wound injury in IL-1R1 null mice, which would contribute to decreased edema and free radical production.

Touzani et al. observed similarities in infarct volumes between WT and IL-1R1 null mice after middle cerebral artery occlusion. They hypothesized the involvement of other putative mediators of neuronal death, such as glutamate, nitric oxide, tumor necrosis factor α, and other free radicals or the existence of an additional IL-1 receptor. Two more likely explanations for the discrepant results between their study and ours are as follows: (1) the mice in this study were subjected to a milder insult than was used by Touzani et al.
opening a possibility that additional tissue-damaging mechanisms involved in the more severe insult could have masked the protection afforded by deleting the IL-1R1; and (2) Touzani et al. used IL-1R1 null mice on a C57BL/6/SV129 mixed background, whereas we used mice that had been backcrossed 9 generations onto a C57BL/6 background.

Conclusion

We conclude that abrogating IL-1R1 signaling could protect brain cells subsequent to a mild stroke by preserving energy status and interfering with chemokine production, as well as by directly interfering with cytokine activation of immune cells. Deficient IL-1R1 signaling also may also diminish iNOS production. The results presented here strongly suggest that therapies targeted to antagonize IL-1R1 signaling will likely prove efficacious in preventing neuronal damage subsequent to mild stroke.

References

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doi: 10.1161/01.STR.0000182255.08162.6a

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