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Blocking receptor for advanced glycation end-products (RAGE) or toll like receptor 4 (TLR4) prevents posttraumatic epileptogenesis in mice

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Abstract

Objective—Effective treatment for the prevention of posttraumatic epilepsy is still not available. Here, we sought to determine whether blocking receptor for advanced glycation end-products (RAGE) or toll like receptor 4 (TLR4) signaling pathways would prevent posttraumatic epileptogenesis.

Methods—In a mouse undercut model of posttraumatic epilepsy, daily injections of saline, RAGE monoclonal antibody (mAb), or TAK242, a TLR4 inhibitor, were made for 1 week. Their effects on seizure susceptibility and spontaneous epileptic seizures were evaluated with a pentylenetetrazol (PTZ) test in 2 weeks and with continuous video and wireless electroencephalographic (EEG) monitoring between 2–6 weeks after injury, respectively. Seizure

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Conflict of interest

All authors confirm that there is no competing financial conflict of interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

susceptibility after undercut in RAGE knockout mice was also evaluated with the PTZ test. The lesioned cortex was analyzed with immunohistology.

Results—Undercut animals treated with RAGE mAb or TAK242 showed significantly higher seizure threshold than saline-treated undercut mice. Consistently, undercut injury in RAGE knockout mice didn't cause a reduction in seizure threshold in the PTZ test. EEG and video recordings revealed a significant decrease in the cumulative spontaneous seizure events in RAGE mAb or TAK242 treated group ($p < 0.001$, when the RAGE mAb or TAK242 group is compared with the saline group). The lesioned cortical tissues of RAGE mAb or TAK242 treated undercut group showed higher neuronal densities of Nissl staining and higher densities of GAD67-immunoreactive interneurons than the saline treated undercut group. Immunostaining to GFAP and Iba-1 revealed lower densities of astrocytes and microglia in cortex of the treatment groups, suggesting reduced glia activation.

Significance—RAGE and TLR4 signaling are critically involved in posttraumatic epileptogenesis. Blocking these pathways early after traumatic brain injury is a promising strategy for preventing posttraumatic epilepsy.

Keywords

Posttraumatic epilepsy; undercut; receptor for advanced glycation end-products (RAGE); toll like receptor 4 (TLR4); neuroinflammation

Introduction

Posttraumatic epilepsy (PTE) develops in 5–53% of patients of traumatic brain injury (TBI) with various injury types and severities [1, 2]. It is poorly controlled by currently available antiepileptic drugs [3] and constitutes one of the major conditions that compromise functional outcome and quality-of-life in patients with TBI [4]. Although the existence of a latent period of varying duration between TBI and seizure onset provides an opportunity for therapeutic intervention for its prevention [2, 5], current therapeutic strategies are not successful [6].

Recent studies suggest that activation of the toll-like receptor 4 (TLR4) or receptor for advanced glycation end-products (RAGE) by high mobility group box-1 (HMGB1) is involved in acquired epileptogenesis [7, 8]. HMGB1 is a nuclear DNA-binding protein that also functions as an endogenous danger signaling molecule and plays a key role in inducing inflammatory response [9]. TBI, ischemia, and seizures all cause HMGB1 release and increase TLR4 expression in brain [8, 10]. Upregulation of HMGB1, RAGE and TLR4 happens in both epileptogenic human and mouse tissues [11, 12]. Administering HMGB1 or TLR4 agonists such as lipopolysaccharide (LPS) results in neuronal hyperexcitability and augmentation of seizure activity [8, 13]. Conversely, neutralizing HMGB1 or eliminating the responsive receptors reduces both acute and chronic recurrent seizures induced by kainic acid injection [8, 13]. Inhibition of TLR4 also retards seizure precipitation as well as reduces acute and chronic seizure recurrence [11]. Furthermore, treatment with a neutralizing HMGB1 antibody inhibits TBI-induced brain edema, protects compromised blood-brain barrier (BBB), and suppresses inflammatory molecule expression during the

acute stage following TBI [14]. Evidence from these studies supports a critical role of RAGE and TLR4 pathways in both neuroprotection after TBI and acquired epileptogenesis. However, the roles of TLR4 and RAGE signaling in posttraumatic epileptogenesis is not clear, particularly, the effect of blocking these signaling pathways on preventing chronic PTE has not been specifically tested. Here we explored the effect and cellular mechanism of blocking TLR4 and RAGE signaling on PTE development in a mouse undercut model. Our results indicated that inhibiting the RAGE or TLR4 pathway significantly increased seizure threshold, decreased the frequency of chronic spontaneous epileptic seizures, and improved neuronal survival and gliosis. These findings support blocking TLR4 and RAGE signaling as an effective strategy for the prevention of PTE.

Materials and methods

Animals and experimental paradigm

C57BL/6 mice and RAGE knockout (RAGE^{-/-}) mice (equal male and female) were housed at most 5 per cage in a temperature- and humidity-controlled animal facility on a 12-hour light/dark cycle, with food and water supplied *ad libitum*. All procedures were approved by the Animal Care and Use Committee of the Institutional Guide for the Care and Use of Laboratory Animals at Indiana University School of Medicine (IUSM).

The study was composed of 4 experiments as described below:

Experiment I: 59 C57BL/6 wild-type (WT) mice at the age of 2 months received undercut or sham surgeries. They received daily vehicle (normal saline), RAGE monoclonal (RAGE mAb, 10 mg/kg, *i.p.*, Abbvie Laboratories, Deerfield, IL) or TAK242, a TLR4 inhibitor (3 mg/kg, *i.p.*, Apexbio Technology LLC, TX) injection for 1 week starting on the next day after surgery. At 2 weeks after the injury, a PTZ test was performed on these animals.

Experiment II: 16 RAGE^{-/-} mice (a kind gift from Dr. Lilian Plotkin at the IUSM) and 20 C57BL/6 mice at the age of 2 months received undercut or sham surgeries. At 2 weeks after the injury, a PTZ test was performed on them.

Experiment III: 16 mice at the age of 9–10 months received undercut or sham surgeries. The undercut animals received daily vehicle, RAGE mAb, or TAK242 injection for 1 week after surgery. At 4 weeks after undercut surgery, they were sacrificed for immunostaining.

Experiment IV: Because older undercut mice are found to have higher probability of developing chronic PTE than younger mice [15, 16], we used old mice for EEG recording to better reveal the effect of the treatment. 21 mice at the age of 9–10 months received undercut surgery. After the animals received daily vehicle, RAGE mAb, or TAK242 injection for 1 week starting on the next day after surgery, they were implanted with a transmitter at day 10 after injury and continuous video and wireless EEG recordings were performed 2–6 weeks after injury.

Undercut surgery and drug treatment

Undercut injury was made as previously described [17]. Briefly, animals were anesthetized with ketamine/xylazine (87.7/12.3 mg/kg, *i. p.*) and fixed on a stereotaxic apparatus. After a midline incision of the scalp and creation of a rectangular window on the left skull, an undercut lesion was made using a device to cut through the cortex and the underneath white matter of the sensorimotor cortex.

All reagents were freshly prepared on the day of use. The neutralizing monoclonal RAGE antibody (RAGE mAb, 11E6) was generated against the murine C2-domain of RAGE [18]. On the next day after undercut or sham surgery, the mice received daily intraperitoneal injection of normal saline or drug treatment for one week. We used 10 mg/kg (*i.p.*) of mouse RAGE-mAb based on a previous study showing that 1–10 mg/kg (*i.p.*) RAGE mAb was effective for inflammatory pain [19] and 3 mg/kg of TAK242 based on previous studies showing effectiveness of this dose for blocking TLR4 signaling in a mouse cerebral ischemia model [20]. Because HMGB1 in cytoplasm peaks in 3 days after TBI and decreases to normal level at day 7, we decided to treat the mice for one week [15].

Detection of systemically injected RAGE mAb in mouse brain

To test if intraperitoneally injected RAGE mAb could pass through the BBB to enter brain tissue, 3 undercut mice received daily injection of 10 mg/kg RAGE-mAb (*i.p.*) for 3 days after injury. Then they were perfused and the brains were sectioned at 30 μ m. After rehydration and blocking, the brain sections were incubated with a goat anti-mouse IgG-Cy3 diluted in a blocking buffer at 4°C overnight. After the sections were processed with DAPI staining, they were washed in PBS and mounted on glass slides for imaging.

Pentylenetetrazol (PTZ) test

A pentylenetetrazol (PTZ) test was made at 2 weeks after undercut surgery using a Modified Racine's scale [21] and similar to our previous studies [22]. A mouse was placed in a transparent box for 15 minutes to calm down, then an initial subconvulsive dose of PTZ (20 mg/kg for Fig. 1B or 30 mg/kg for Fig. 1C and Fig. 2, *i.p.* Sigma-Aldrich, MO) was injected and the mouse was observed for 15 minutes. Thereafter, additional doses of 10 mg/kg of PTZ were given every 15 minutes until a convulsive seizure was observed. The total time from first PTZ injection to convulsive seizure and cumulative dose of PTZ injected were recorded.

Immunofluorescent staining

Mice were perfused and the brains were processed for immunofluorescence using antibodies against glutamate decarboxylase (GAD) 67, glial fibrillary acidic protein (GFAP), and ionized calcium binding adaptor molecule 1 (Iba1) for identifying cortical GABAergic interneurons, astrocytes, and microglia, respectively.

After imaging, 3–5 brain slices containing the undercut lesion from each mouse were selected for cell counting. Nissl(+), GAD67(+), Iba-1(+) cell were manually and blindly counted in 0.04 mm² squares at a distance of 200 μ m to the edge of transcortical lesion. GFAP(+) astrocytes were quantified using NIH ImageJ and expressed as areal

density. Detailed immunostaining method and data analysis can be found in the Supporting Information.

Continuous video-EEG recording and data analysis

Mice were monitored for spontaneous seizures from 2 to 6 weeks after injury using continuous video-EEG recording. A telemeter system was used for continuous EEG recording (Epoch system, Epitel Inc, Salt Lake City, Utah) [23]. At 10 days after undercut surgery, a miniature two-channel transmitter was installed onto the skull (Fig. 3A) and EEG recording at a sampling rate of 500 Hz was made for 4 weeks. A digital video camera was used to simultaneously monitor the behavior of the mice. The EEG traces were manually and blindly analyzed. Details and data analysis are provided in the Supporting Information.

Statistical analysis

The data of experiment *I* and *III* were analyzed using One-way ANOVA followed by a Newman-Keuls test, the data of experiment *II* were analyzed using Two-way ANOVA followed by a Bonferroni test, and the data of experiment *IV* were compared among all groups using non-parametric Kruskal-Wallis or Kolmogorov-Smirnov test. Data are presented as mean \pm SD, and the significance level was set as $p < 0.05$.

Results

Blocking RAGE or TLR4 signaling reduced seizure susceptibility in undercut mice

We first detected the expression of HMGB1, TLR4 and RAGE under normal condition and in 3 days after undercut. We found that clusters of HMGB1 puncta in nuclei of sham mice were replaced by larger and homogeneous staining of cell bodies of neurons, astrocytes, and microglia after undercut, suggesting a translation of HMGB1 from nuclei to cytoplasm (Fig. S1 A). The expressions of TLR4 and RAGE in cortex were pretty low or undetectable in sham mice, but they increased greatly after undercut in neurons, microglia, and endothelial cells, but not in astrocytes (Fig. S1 B–C). Expressions of RAGE or TLR4 were seen only in a small number of astrocytes in white matter next to the undercut lesion area (Fig. S2). We then tested whether intraperitoneal injection of a RAGE mAb would pass through the BBB to enter the brain parenchyma. As shown in Fig. 1A, RAGE mAb was detected in neurons and microglia of the injured mouse cortex after daily injection of RAGE mAb for 3 days after undercut, with a higher signal level particularly in regions surrounding the undercut site, suggesting that the injured cortex is accessible to systemic injection of the RAGE mAb.

To efficiently evaluate seizure susceptibility after TBI and the effect of blocking RAGE and TLR4 signaling, we used a PTZ test which is commonly used for measuring the efficacy of antiepileptic drugs [21, 24]. The mice with chronic undercut injury had a significantly lower PTZ dose (40.7 ± 12.7 mg/kg) and a shorter latency (21.9 ± 18.2 min) for the induction of tonic-clonic seizure than the sham animals (78.3 ± 15.9 mg/kg and 78.8 ± 25.8 min, both $p < 0.001$) (Fig. 1B). Undercut animals treated with RAGE mAb for 1 week after undercut showed a significantly higher PTZ dose (54.0 ± 18.1 mg/kg) and a longer latency (41.2 ± 27.6 min) for seizure induction than those receiving vehicle injection (Fig. 1B. $p < 0.05$, UC-NS vs UC-RAGE mAb).

Similarly, we examined the effect of blocking TLR4 with TAK242 [20, 25], on seizure susceptibility. Undercut mice receiving TAK242 treatment after injury for 1 week had a significantly higher PTZ dose (100.0 ± 21.0 mg/kg) and a longer latency (125.9 ± 32.5 min) for seizure induction than those receiving vehicle injection (71.7 ± 17.2 mg/kg and 82.1 ± 25.9 min) (Fig. 1C, $p < 0.05$, UC-NS vs UC-TAK242). These results suggest that blocking RAGE or TLR4 for 1 week after brain injury prevented the development of high seizure susceptibility in chronic undercut mice.

RAGE knockout mice did not develop higher seizure susceptibility after undercut injury

To determine whether RAGE signaling plays a critical role in PTE, we tested whether deletion of RAGE would affect development of higher seizure susceptibility after TBI in RAGE knockout mice (Fig. 2). Similarly, the WT undercut mice had a significantly lower PTZ dose (59.0 ± 32.1 mg/kg) and a shorter latency (64.2 ± 47.9 min) for seizure induction than sham animals (97.0 ± 29.1 mg/kg and 124.3 ± 47.3 min) in PTZ test (Fig. 2, $p < 0.01$, WT-Sham vs WT-UC). In contrast, RAGE^{-/-} undercut mice had no significant differences in PTZ dose (93.8 ± 18.5 mg/kg) and latency (115.1 ± 29.3 min) for seizure induction from the sham RAGE^{-/-} mice (100.0 ± 19.3 mg/kg and 126.6 ± 27.7 min, $p > 0.05$ for both). When the WT and RAGE^{-/-} mice were compared, the differences in both PTZ dose and latency to seizure between the two sham groups were insignificant, but the differences between the two undercut groups were significant (Fig. 2, $p < 0.05$, WT-UC vs RAGE^{-/-}-UC). This outcome was consistent with the RAGE mAb treatment experiment, indicating that RAGE signaling is essential for posttraumatic epileptogenesis.

RAGE mAb or TAK242 treatment after undercut prevented posttraumatic epileptogenesis

To further confirm whether early treatment after brain injury with RAGE mAb or TAK242 would have prophylactic effect on posttraumatic epileptogenesis, we used continuous video and wireless EEG recording to monitor seizure activity for 4 weeks in the undercut mice. While sham mice did not develop spontaneous EEG seizures (Fig. S3, $n = 4$), undercut mice showed spontaneous seizures in several weeks after injury [22], characterized as high amplitude paroxysmal discharges in both ipsilateral and contralateral hemispheres (Fig. 3B).

In vehicle-treated UC mice, all (6 of 6) developed chronic spontaneous seizures after injury, with a latency of 19.7 ± 0.6 days and a seizure frequency of 0.46 ± 0.30 seizures/day. In RAGE mAb or TAK242 treated mice, 5 of 7 (71.4%) or 4 of 6 (66.7%) developed spontaneous seizures with a latency of 21.0 ± 1.4 days or 22.0 ± 3.0 days and seizure frequencies of 0.18 ± 0.23 or 0.09 ± 0.15 seizures/day, respectively (Fig. 3C). Although there were no significant differences among the three groups in the percentage of epileptogenic animals, the TAK242 group has a significantly lower frequency of chronic spontaneous seizures (Fig. 3C, $p < 0.05$ when compared with the UC-NS group). A plot of cumulative seizures over time indicated significantly lower seizure events in both of the RAGE mAb and TAK242 groups, with the TAK242 group being the lowest (Fig. 3D, $p < 0.001$ comparing RAGE mAb or TAK242 with vehicle-treated group, $p < 0.01$ between RAGE mAb and TAK242-treated groups). In addition, neither RAGE mAb nor TAK242 treatment affected the average duration of seizure events (Fig. 3C, $p > 0.05$ for both comparisons).

Treatment with RAGE mAb or TLR4 antagonist TAK242 reduced gliosis in undercut cortex

TLR4 and RAGE signaling is known to initiate innate immune response [26]. To determine whether blocking their signaling after brain injury would prevent glial cell activation, we examined the densities of astrocytes and microglia in the injured cortex by using immunostaining against GFAP and Iba-1 as markers for astrocytes and microglia respectively. In the uninjured cortex, the level of GFAP expression was extremely low (Fig. 4A–B). Undercut caused a dramatic increase in GFAP immunoreactivity and larger cell bodies of astrocytes with elongated processes in the injured cortex in 4 weeks after injury, which were significantly decreased or normalized after RAGE mAb or TAK242 treatment (Fig. 4A–B, $p < 0.05$, UC-NS vs UC-RAGE mAb or UC-TAK242). Similarly, undercut caused a dramatic increase in Iba-1 immunoreactivity and microglia with swollen cell bodies with less ramification in the injured cortex in 4 weeks after injury, which were significantly decreased or normalized after RAGE mAb or TAK242 treatment (Fig. 4C–D, $p < 0.01$, 0.001 , UC-NS vs UC-RAGE mAb or UC-TAK242). Together, these results suggest that treatment with RAGE mAb or TAK242 after undercut inhibited astrocytes and microglia activation in injured cortex.

RAGE mAb or TAK242 treatment had neuroprotective effect in undercut cortex

To explore the effect of blocking RAGE or TLR4 on neuronal loss after TBI, we examined the density of cortical neurons in lesion area after chronic undercut in Nissl-stained sections. The undercut caused a significant loss of cortical layer V neurons within the lesion area (Fig. 5, $p < 0.01$, compared with Sham group); treatment with RAGE mAb or TAK242 for 1 week resulted in reduced losses of cortical layer V neurons, although the neuron numbers were still lower than that of the sham animals (Fig. 5, $p < 0.05$, UC-NS vs UC-RAGE mAb or UC-TAK242). The results suggest a protective effect of blocking RAGE or TLR4 after injury on cortical neurons in undercut mice.

RAGE-mAb or TAK242 treatment restored interneuron in undercut cortex

Loss of inhibitory neurons is observed in models of acquired epilepsy including PTE [27]. To determine the effect of blocking RAGE and TLR4 on the preservation of inhibitory neurons, we used immunostaining to GAD67 to label cortical GABAergic interneurons and counted their densities. The density of GAD67+ neurons was significantly lower in the lesion area of undercut mice than that of sham mice (Fig. 6, $p < 0.001$, UC-NS vs Sham), but they were significantly higher in the groups treated with RAGE mAb or TAK242 after undercut than the undercut group treated with saline, even though they were still lower than the sham group (Fig. 6, $p < 0.05$ UCNS vs UC-RAGE mAb or UC-TAK242). This finding suggests a protective effect of blocking RAGE or TLR4 on cortical inhibitory neurons in undercut mice.

Discussion

Herein, we show that blocking RAGE or TLR4 pathway after TBI resulted in elevated threshold for seizure induction and reduced chronic spontaneous seizure events in the undercut model, suggesting that these pathways participate in posttraumatic epileptogenesis. The treatment ameliorated the loss of cortical neurons including interneuron loss, suggesting

a neuroprotective effect of these treatments. We also observed fewer cortical astrocytes and microglia in the treated mice, implying the involvement of RAGE and TLR4 signaling in astrogliosis and microgliosis in response to TBI.

HMGB1 is constitutively expressed and localized in the nucleus in many cell types including glial cells and neurons. After brain injuries and seizures, HMGB1 becomes acetylated and translocates from the nucleus to the cytoplasm. It can then be released from necrotic or activated immune cells into extracellular space and triggers inflammatory responses by binding multiple receptors including RAGE and TLR4 [9]. Previous studies have demonstrated that HMGB1 signaling contributes to the generation of epilepsy through activating RAGE and TLR4 in chemoconvulsant models such as pilocarpine or kainic acid and that interventions targeting to this pathway could retard seizure precipitation and decrease the recurrence of acute and chronic seizures [13, 28]. The expression of TLR4 in human epileptogenic tissue as well as in a mouse model of chronic seizures was increased [11]. Cortical application of lipopolysaccharide (LPS), an agonist of TLR4, produced focal epileptiform discharges [29]. RAGE or TLR4 KO mice had lower seizure susceptibility in acute seizure assessment by kainic acid, as well as lower spontaneous seizure frequency in a chronic temporal lobe epilepsy model [12, 13]. However, the specific role of RAGE and TLR4 in posttraumatic epileptogenesis and the effect of blocking them on PTE prevention are largely unknown.

The undercut model is a penetrating PTE model with increased seizure susceptibility and chronic spontaneous epileptic seizures [22, 30]. We showed the translocation of HMGB1 and increased expressions of TLR4 and RAGE in 3 days after undercut in neurons and microglia, but not astrocytes (Fig. S1), which are consistent with earlier findings in Alzheimer's disease and brain ischemia [31, 32]. RAGE or TLR4 has been shown to play pivotal roles in brain injury induced pathological processes that are associated with epileptogenesis, such as BBB disruption, neuronal loss, and neuronal excitability enhancement [33]. In a global cerebral ischemia model, the number of surviving neurons in hippocampus was much higher in RAGE^{-/-} mice than wild-type mice, supporting the contribution of RAGE to ischemia induced neuronal death [34]. Consistently, administration of RAGE antagonist FPS-ZM1 significantly ameliorated BBB damage, brain edema and motor dysfunction in an intracerebral hemorrhage model of stroke [35]. TLR4 is also involved in the development of brain injury. Silencing TLR4 by application of TLR4 short hairpin RNA (shRNA) ameliorated neuroinflammatory response, alleviated hippocampal neuronal damage, and improved neurological deficits after TBI [36]. Treatment with TLR4 antagonist TAK242 before injury attenuated TBI caused neuronal loss, brain edema and neurobehavioral impairment in rats [37]. Concussive brain injury was found to enhance excitability of hippocampal dentate gyrus, while TLR4 antagonists decreased and a TLR4 agonist increased afferent-evoked dentate excitability 1 week after brain injury [38]. These studies provide clues that RAGE and TLR4 may contribute to PTE. Consistently, we found that blocking RAGE either by a RAGE mAb or genetic deletion and blocking TLR4 with TAK242 prevented the elevation of seizure susceptibility caused by undercut (Fig. 2) and decreased spontaneous seizure events in undercut mice (Fig. 3). These results suggest that RAGE and TLR4 contribute to posttraumatic epileptogenesis in the mouse model of PTE and blocking them has prophylactic effect. However, although TLR4 blockade with

TAK-242 (Fig. 1C) or RAGE knockout (Fig. 2) resulted in about similar levels of seizure susceptibility in undercut mice as sham or wild-type mice in the PTZ test, the results do not necessarily suggest that TLR4 or RAGE signaling is the only mechanism responsible for posttraumatic epileptogenesis. It is possible that PTZ test, while a valuable and efficient surrogate for assessing seizure threshold, may not sensitively reflect other pathological features of posttraumatic epilepsy (as compared to Fig. 3C–D) and that other mechanisms may work in parallel or in synergy with the TLR4/RAGE signaling.

Inhibiting glial activation may be a mechanism in our observed prophylactic effect. Gliosis is a common feature in PTE. After brain trauma, local microglia and astrocytes are primed by cellular debris released by damaged cells within the lesion areas [39], and RAGE and TLR4 expressed on astrocytes and microglia are activated by danger signals like HMGB1, S100 proteins, amyloid- β , and other damage-associated molecular patterns (DAMPs) molecules [40, 41]. Once activated, RAGE or TLR4 triggers a series of intracellular signaling events resulting in transcription of inflammatory genes such as IL-1 β via NF- κ B, leading to phosphorylation of NMDA-NR2B receptors and enhanced excitability of neural network and seizure generation [11, 42]. The levels of RAGE and TLR4 in astrocytes and microglia are both up-regulated in animal models of epilepsy and brains of human epilepsy patients [12]. In our study, either anti-RAGE-mAb or TLR4 antagonist TAK242 reduced gliosis, which is in agreement with previous studies demonstrating that pretreatment with TAK242 markedly inhibited ischemic reperfusion induced astrocytic and microglial activation in spinal cord [43]. Our results suggest inhibition of RAGE or TLR4 may attenuate the contribution of astrocytes and microglia in development of PTE.

Another possible effect mechanism of blocking RAGE or TLR4 signaling is through neuroprotection. TBI initiates a cascade of pathological changes including neuroinflammation that persists for weeks, with evidence of widespread neuronal loss during posttraumatic epileptogenesis [44]. TAK242 was shown to ameliorate neuronal loss caused by TBI [25], which is consistent with our results demonstrating neuronal restoration in TAK242-treated undercut mice. Consistent with the observation of improved neuronal survival with deletion of RAGE in an ischemic brain injury model [45], our data also showed that treatment with an anti-RAGE mAb after brain injury improved cortical neuronal survival.

A number of studies reported that cortical inhibitory neurons are more vulnerable to TBI with evidence of considerable interneuron loss in lesioned brain regions [46, 47] and that inflammatory signaling such as TNF α may play a major role in modulating interneuron damage after TBI [48]. Decreased frequency of spontaneous inhibitory postsynaptic currents and increased frequency of spontaneous excitatory postsynaptic currents have been found in both controlled cortical impact (CCI) model and undercut model [30, 49], which supports a role of loss of inhibition in posttraumatic epileptogenesis. We found that blocking RAGE or TLR4 improved GAD67+ neuron survival, which may contribute to the reduction of seizure susceptibility and prevention of epileptogenesis by restoring GABAergic neurons and regulating excitation/inhibition balance in the undercut cortex. Although it is unclear about the role of RAGE and TLR4 on interneuron survival, activation of TLR4 has been shown to attenuate GABA synthesis and postsynaptic GABA receptor activities in the spinal

dorsal horn [50]. Whether the restored density of cortical interneurons would translate into a normal network inhibition requires electrophysiological evaluation in future.

Conclusion

We demonstrated that specific blockade of RAGE and TLR4 pathways early after TBI is effective in decreasing seizure susceptibility and reducing the frequency of chronic epileptic seizures, suggesting a prophylactic effect on posttraumatic epileptogenesis. The cellular mechanism of this effect is associated with promoting survival of cortical neurons, particularly GABAergic interneurons, and inhibiting gliosis. Our study provides new insights for understanding the role of RAGE and TLR4 in PTE and for preventing posttraumatic epileptogenesis by targeting this molecular pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key points

- Mice with undercut had higher seizure susceptibility, which was prevented by early treatment after injury with RAGE antibody or a TLR4 inhibitor TAK242;
- Video/EEG monitoring showed that either of the treatment significantly reduced the chronic spontaneous seizure events between 2–6 weeks after injury;
- RAGE knockout mice after undercut did not develop higher seizure susceptibility;
- Treatment with RAGE antibody or TAK242 resulted in higher densities of cortical neurons and interneurons and lower densities of astrocytes and microglia.

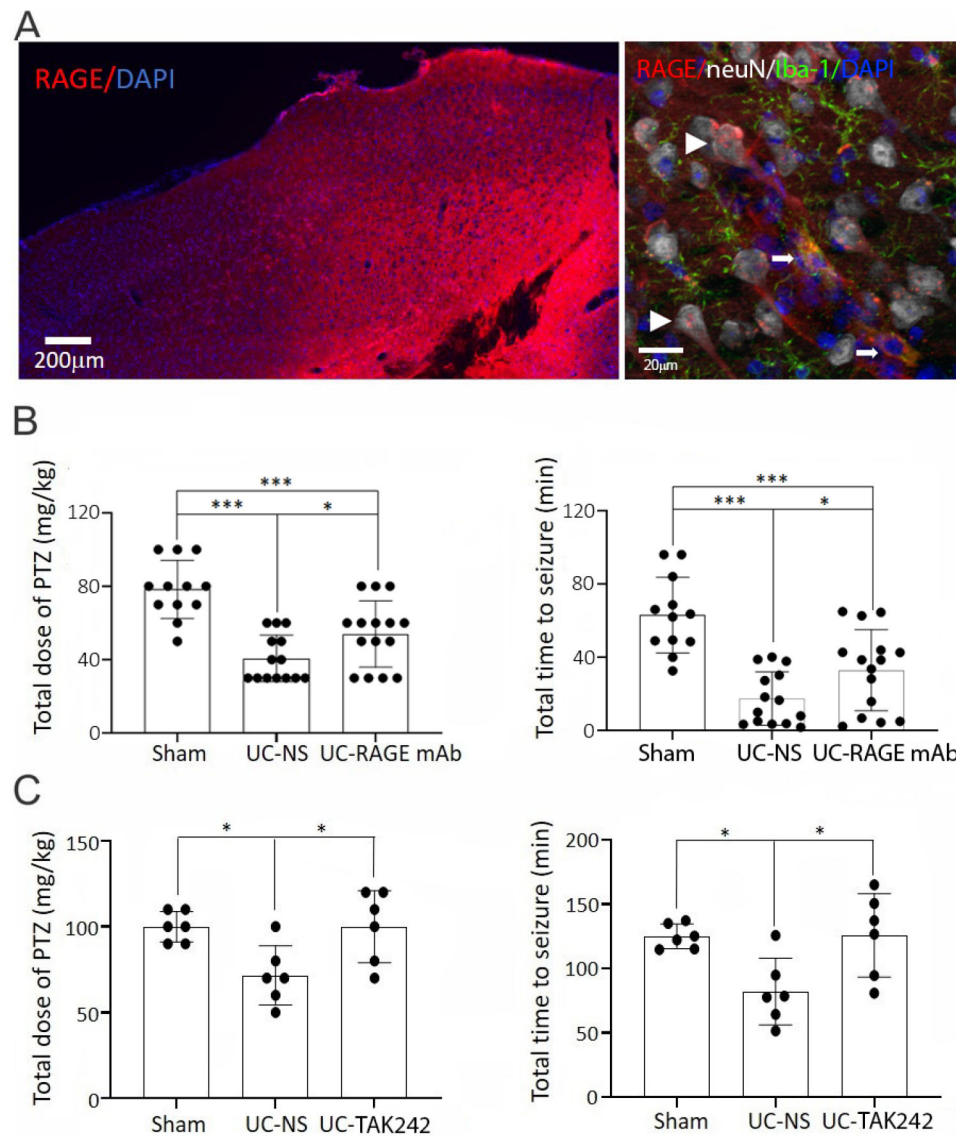


Figure 1.

Early blockade of receptor for advanced glycation end-products (RAGE) or toll like receptor 4 (TLR4) after undercut (UC) reduced seizure susceptibility. **A**. Left: A representative image showing the distribution of the RAGE mAb (in red) in the undercut cortex after its intraperitoneal injection. Right: Quadruple staining for detecting the binding of RAGE mAb to neurons and microglia. The strong RAGE mAb fluorescent signals on the surface of cortical pyramidal neurons (neuN positive, arrowheads) and microglia (Iba-1 positive, arrows) indicate binding of the antibody preferentially on membranes of these cells. **B-C**. Undercut mice treated with vehicle (normal saline; NS) showed lower seizure threshold, as reflected by a lower accumulative dose of pentylenetetrazol (PTZ) and a shorter latency for PTZ-induced acute seizure than the sham animals. In contrast, the mice treated with RAGE mAb (**B**) ($n = 12-15$) or TAK242 (TLR4 inhibitor, $n = 6$) (**C**) after undercut injury for 1 week had higher cumulative doses of PTZ and longer latency periods than vehicle group (*:

$p < 0.05$; *** $p < 0.001$; One-way ANOVA followed by Newman-Keuls test), suggesting increased seizure thresholds.

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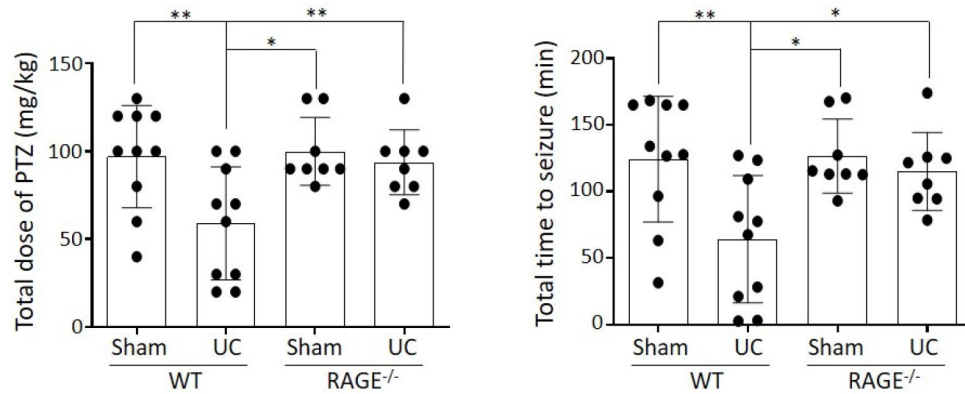


Figure 2.

Receptor for advanced glycation end-products (RAGE) knockout (KO) mice did not develop higher seizure susceptibility after undercut (UC). In contrast to wild type (WT) of undercut mice that had lower cumulative pentylenetetrazol (PTZ) dose and latency for seizure induction, RAGE^{-/-} undercut mice didn't have significant decreases in cumulative PTZ dose and latency for seizure induction ($p > 0.05$, RAGE^{-/-} Sham vs RAGE^{-/-} UC). The RAGE^{-/-} undercut mice also had higher cumulative PTZ dose and higher latency for seizure induction than the WT undercut mice (*: $p < 0.05$, WT-UC vs RAGE^{-/-} UC, Two-way ANOVA followed by Bonferroni tests, $n = 8-10$), suggesting a resistance to TBI-induced epileptogenesis of the RAGE KO mice.

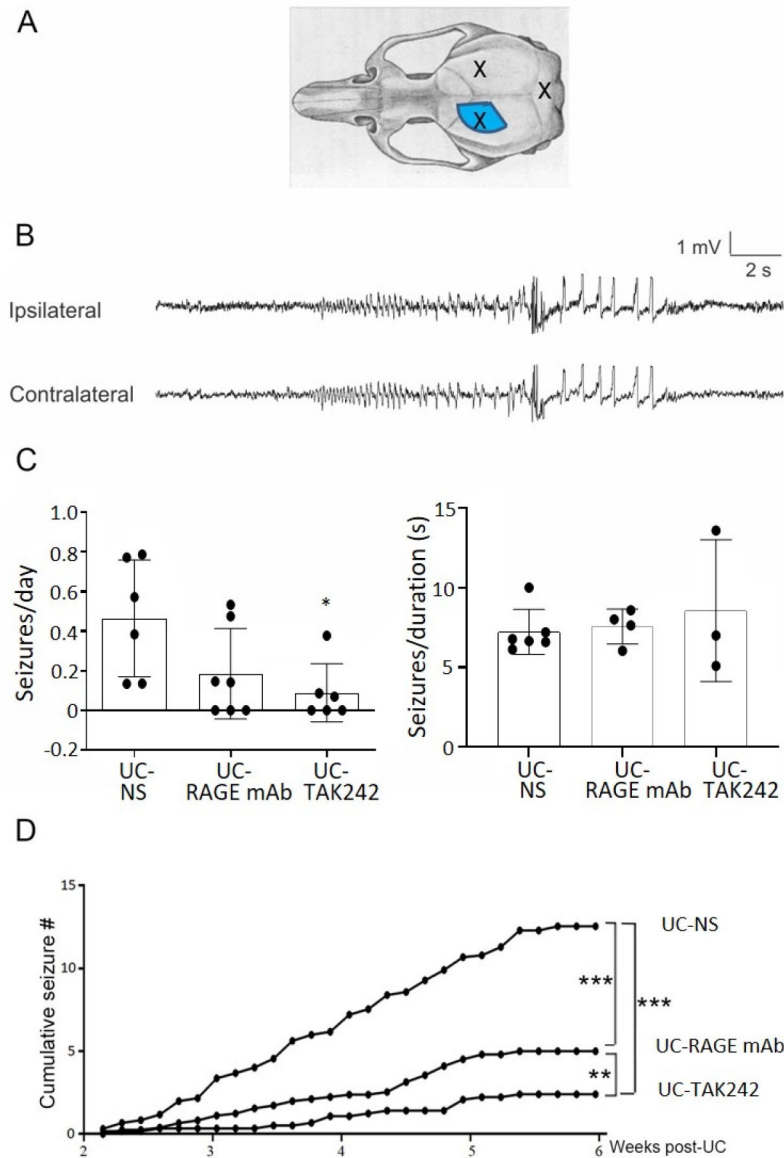


Figure 3.

Early blockade of RAGE or TLR4 after undercut prevented posttraumatic epileptogenesis.

A. A schematic of the placement of electrodes in mouse skull for continuous wireless EEG recording. B. Representative traces of a spontaneous seizure event recorded from cortex ipsilateral and contralateral to traumatic lesion in an undercut mouse. C. Undercut mice receiving early treatment of TAK242 after injury for 1 week had significantly lower frequencies of spontaneous seizure events than the mice treated with vehicle (normal saline; NS) (left) (*: $p < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparisons test). But there were no significant differences in the average durations of seizure events among the 3 groups (right). D. Highly significant decreases in cumulative number of spontaneous seizures in both RAGE mAb and TAK242 groups during the recording period (**: $p < 0.01$; ***: $p < 0.001$, Kolmogorov-Smirnov test, $n = 6-7$).

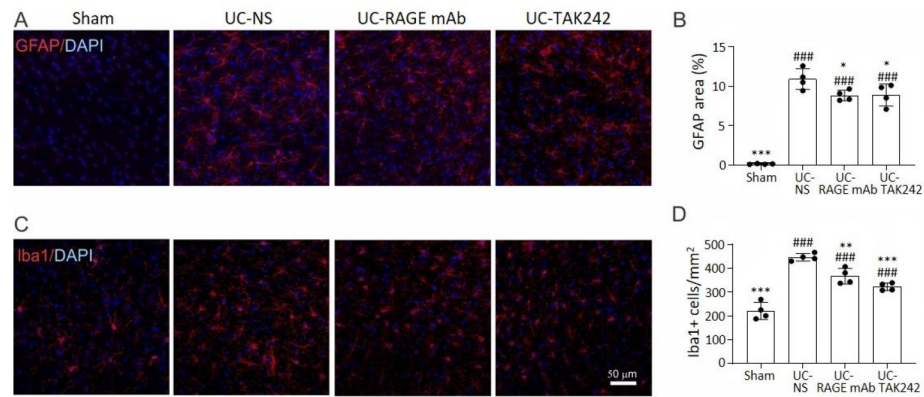


Figure 4.

Early blockade of RAGE or TLR4 after undercut reduced gliosis. A-B. Immunostaining to an astrocyte marker GFAP (red) showed that undercut induced astrocyte proliferation in the lesion area of cortex. Early treatment after undercut with RAGE mAb or TAK242 for 1 week significantly reduced GFAP immunoreactivity (*: $p < 0.05$ and ***: $p < 0.001$, when compared with UC-NS; ###: $p < 0.001$, when compared with sham. One-way ANOVA followed by Newman-Keuls test). C-D. Immunostaining to a microglia marker Iba-1 (red) showed that undercut induced microglia activation in the lesion area of cortex. Early treatment after undercut with RAGE mAb or TAK242 for 1 week significantly reduced the number of microglia cells (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, when compared with UC-NS; ###: $p < 0.001$, when compared with sham, One-way ANOVA followed by Newman-Keuls test, $n = 4$).

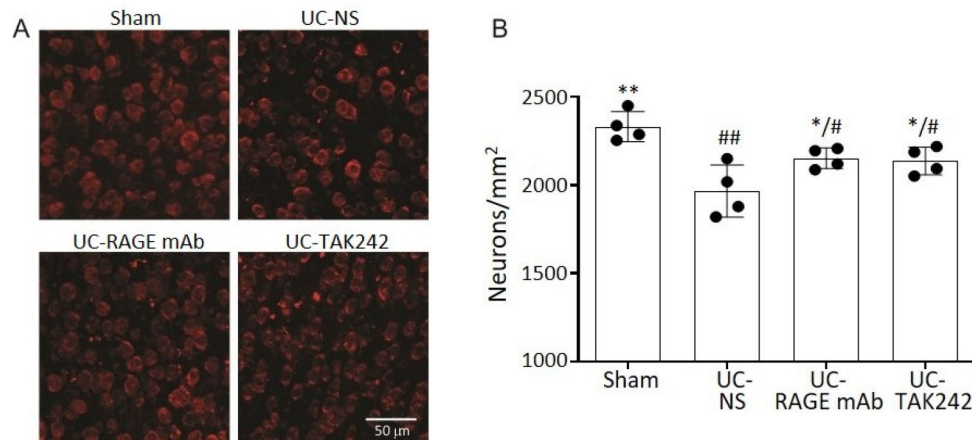


Figure 5.

Early blockade of RAGE or TLR4 after undercut had a neuroprotective effect on the injured cortex. A-B. Fluorescent Nissl staining showed that undercut resulted in a significantly lower neuronal density in the lesioned cortex; early treatment after undercut with RAGE mAb or TAK242 for 1 week significantly increased the neuronal density (*: $p < 0.05$; **: $p < 0.01$, when compared with UC-NS. #: $p < 0.05$; ##: $p < 0.01$, when compared with the sham group. One-way ANOVA followed by Newman-Keuls test, $n = 4$), suggesting a neuroprotective effect of the treatments.

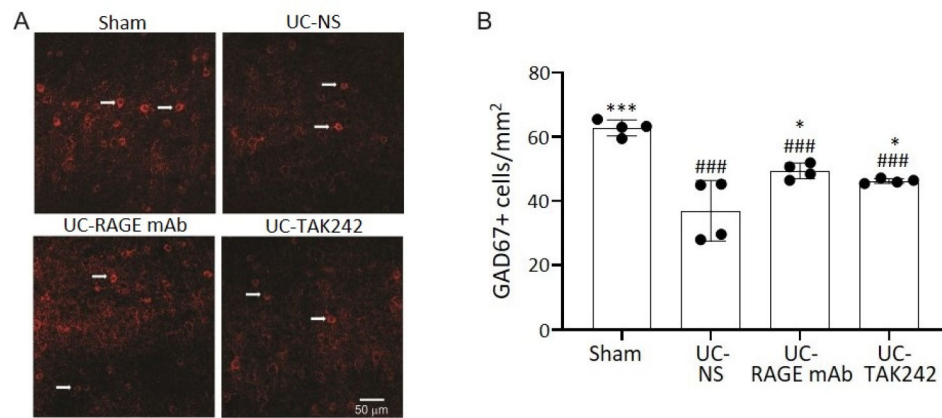


Figure 6.

Early blockade of RAGE or TLR4 after undercut increased the density of interneurons in the injured cortex. A-B. Immunostaining to an interneuron marker GAD67 showed that undercut caused significantly lower density of GAD67(+) neurons in the lesioned cortex. Early treatment after undercut with RAGE mAb or TAK242 for 1 week significantly increased the density of GAD67(+) neurons (*: $p < 0.05$; ***: $p < 0.001$, when compared with UC-NS; ###: $p < 0.001$, when compared with sham, One-way ANOVA followed by Newman-Keuls test, $n = 4$).