FULL-LENGTH ORIGINAL RESEARCH

Molecular alterations underlying epileptogenesis after prolonged febrile seizure and modulation by erythropoietin

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SUMMARY

Purpose: Children who experience complex febrile seizures are at a higher risk of subsequent epileptic episodes, and they may require therapy. This issue can be resolved by interventional studies using molecular targets identified and defined in animal models. In the current study, the molecular changes in the rat brain after febrile seizures were examined throughout the latent period, and erythropoietin was administered as a potentially antiepileptogenic intervention.

Methods: The changes in the expressions of genes that were differentially regulated during the latent period after febrile seizures were categorized into the following four patterns: (1) continuously high (CH); (2) continuously low (CL); (3) rise and fall (RF); and (4) going-up (GU). Erythropoietin was administered immediately after seizure cessation and then once daily for at most 7 days, and spontaneous recurrent seizures and cellular and molecular changes were investigated.

Key Findings: The CH genes were associated with cell cycle and adhesion, whereas the CL genes were related to energy metabolism. Within the category of RF, the largest changes were for genes involved in inflammation, apoptosis, and γ-aminobutyric acid (GABA) signaling. The GU category included genes involved in ion transport and synaptogenesis. Along with an early rise in inflammatory genes, there were substantial increases in brain edema and activated microglia during the early latent period. Erythropoietin reduced the early inflammatory responses and modulated the molecular alterations after febrile seizures, thereby reducing the risk of subsequent spontaneous seizures.

Significance: Erythropoietin treatment may provide a new strategy for preventing epilepsy in susceptible individuals with atypical febrile seizures.

KEY WORDS: Febrile seizure, Epileptogenesis, Epilepsy, Inflammation, Erythropoietin.

Febrile seizures, which occur in association with fevers in children, are the most common type of seizures in young children (Hauser, 1994). Although most febrile seizures appear to be benign, one-third are complex with a prolonged duration or recurrent episodes, and are associated with a risk of subsequent temporal lobe epilepsy (TLE; Sagar & Oxbury, 1987; French et al., 1993). A subgroup of patients with TLE does not become seizure-free with the current medication, and often develop resistance to all available drugs. Therefore, prevention of epilepsy in patients at risk, that is, complex febrile seizure patients, is an important goal of future therapy.

Work on an experimental model has provided much information about the effects of febrile seizures on cell death, network reorganization, plasticity, and epileptogenesis (Sperber et al., 1991; Toth et al., 1998; Chen et al., 1999, 2001; Brewster et al., 2002; Chen et al., 2003; Dubé et al., 2006; Tsai & Leung, 2006; Lemmens et al., 2008). There is also a growing body of evidence to suggest that inflammatory cells and proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α), play significant roles as effectors of the clinical and pathologic features of epilepsy following febrile

seizures (Vezzani et al., 1999; De Simoni et al., 2000). However, few data support the association of the cellular and molecular alterations with long-lasting epileptogenic remodeling. Serial investigations of the latent period at the molecular level can yield insights into the epileptogenic mechanism after a febrile seizure. In the current study, we provide the first large-scale analysis of the molecular changes underlying epileptogenesis after febrile seizures.

Another pertinent problem is whether acute or prophylactic medical intervention might influence the development of later epilepsy following febrile seizures. Preventive or interventional strategies against febrile seizures are limited, primarily because of the lack of knowledge about mechanisms responsible for epileptogenesis and the long latent period needed for these studies. The cytokine hormone, erythropoietin (EPO), plays critical roles as a modulator of inflammation and a neuroprotective agent (Brines et al., 2000; Yatsiv et al., 2005; Savino et al., 2006; Villa et al., 2007). Because pretreatment or posttreatment with EPO significantly suppresses epileptogenesis by inhibiting inflammation and protecting the blood–brain barrier (BBB) in other models of epilepsy (Brines et al., 2000; Uzum et al., 2006; Nadam et al., 2007; Chu et al., 2008), we have hypothesized that (1) EPO antagonizes the molecular alterations following febrile seizure; and (2) EPO suppresses the risk of spontaneous seizures. These hypotheses are critical because they elucidate the relationship of febrile seizures and the subsequent development of epilepsy, and can define novel interventional strategy.

METHODS

Febrile seizure model

We used pups from timed-pregnant Sprague-Dawley rats (Orient, Seoul, South Korea) that were kept together with their littermates, except during experiments. All procedures were performed with institutional approval and in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. We attempted to develop febrile seizure models with more obvious changes to intervene in the epileptogenic process. The two hyperthermia (HT) conditions were initially applied to develop atypical febrile seizure as follows: (1) prolonged HT, with duration of 45 min (45HT); and (2) repeated HT, with duration of 30 min once daily for 3 days (30RHT). However, because the cellular and molecular changes were similar in the 45HT and 30RHT groups (Figs. S2 and 3), the 45HT model was utilized in most experiments. On postnatal day (P) 10, the febrile seizure model was developed using hyperthermia, which was induced by heated air (50–52°C) from a hair dryer as described previously (Baram et al., 1997; Lemmens et al., 2008). The intensity of the air was adjusted to maintain the core temperature at 41–42.5°C. The occurrence of seizures was monitored by two independent observers. Immediately after the HT period, the rats were placed
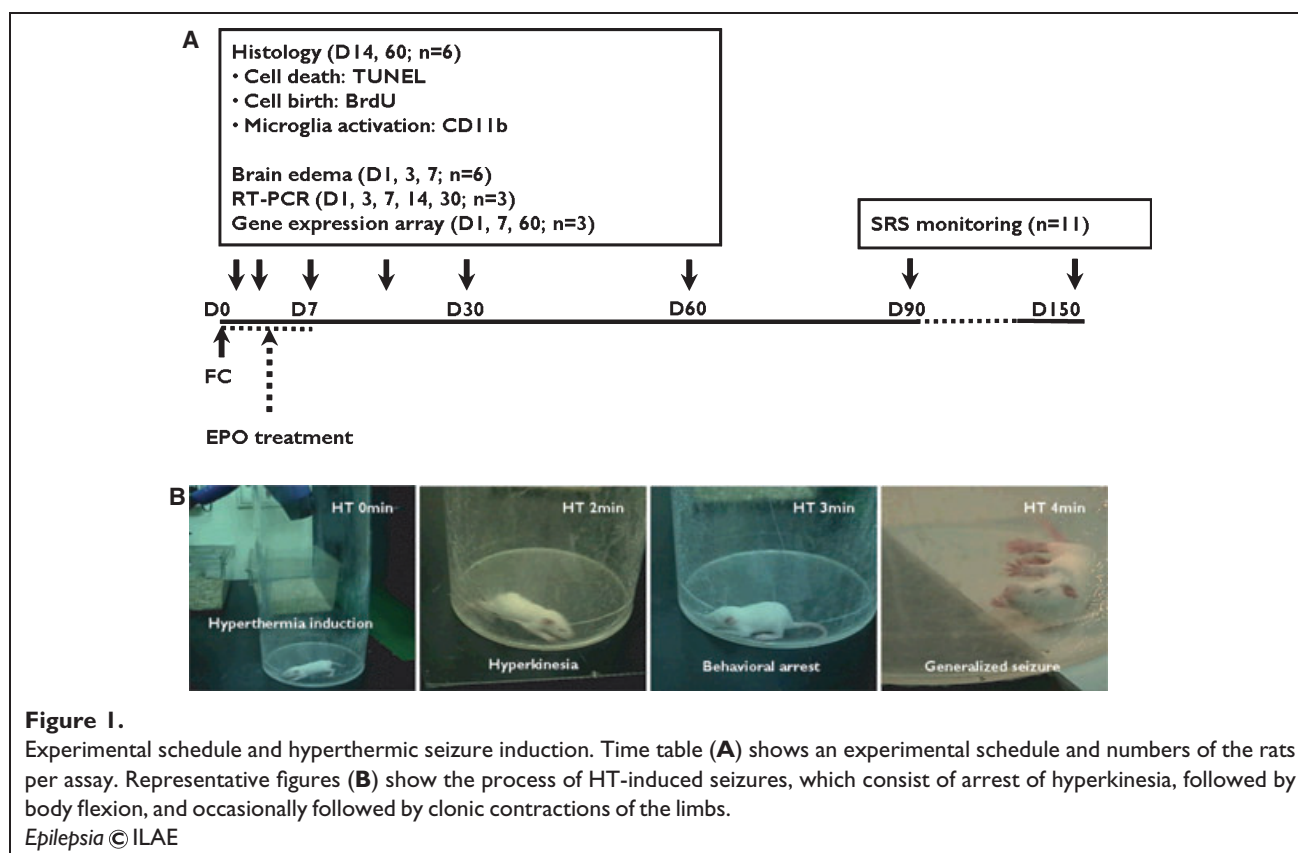
on a cool surface, monitored for 15 min, and then returned to their home cages for rehydration by their mothers. At P21, all pups were weaned and randomly housed (2–3 per cage). A total of 150 immature rats [45HT (n = 141), 30RHT (n = 9)] went through the complete heating and cooling procedures. Rats not receiving HT induction served as normothermia (NT) group (n = 68).

Experimental protocol

The primary strategy was to determine whether EPO can modulate subsequent spontaneous recurrent seizure (SRS) development after febrile seizures. This experiment included 45HT with saline (HT) and 45HT with EPO treatment groups (HT + EPO). Recombinant human EPO (rhEPO, 5,000 IU/kg/day, i.p., diluted in saline; CJ Pharmaceuticals, Seoul, Korea) was administered immediately after seizure cessation and then once daily for at most 7 days, or until sacrifice. The dose of EPO used in the present study was previously determined as the tolerated dose for this route of administration (Lee et al., 2006; Chu et al., 2008). The HT group received the same volume of saline. We monitored chronic animals by video–electroencephalography (EEG), and compared the SRS characteristics in the HT and HT + EPO groups (n = 11 per group). The second strategy was to compare the molecular and cellular changes between the NT, HT, and HT + EPO groups. For analysis of cell genesis, a 5-bromo-3′-deoxyuridine (BrdU; Roche Diagnostics Corp., Indianapolis, IN, U.S.A.) was injected intraperitoneally (100 mg/kg, dissolved in 0.9% saline) once daily for 7 consecutive days beginning on the day of HT induction. The timetable for the experimental schedule is shown in Fig. 1A.

Long-term video-EEG monitoring

Chronic SRSs were analyzed with a combined video-EEG system (Comet XL; Astro-Med, Inc., Warwick, RI, U.S.A.) for 7 consecutive days on D90 and D150 post-HT. Epileptic rats (n = 11 per group) were unilaterally implanted with a depth electrode into the dorsal hippocampus and a cortical electrode over the frontoparietal cortex 2–3 weeks before monitoring as described previously (Chu et al., 2008). EEG data were recorded using long flexible cables (Plastics One, Roanoke, VA, U.S.A.) in freely moving, nonanesthetized rats, and analyzed off-line using PSG Twin 4.2 (Astro-Med, Inc.) by independent observers for group allocation. EEG parameters of electrographic seizures included a high-amplitude (>2 × baseline), high-frequency (>8 Hz) discharge in the hippocampus, cortex, or both, lasting for at least 6 s (Fig. 2A). If an electrographic seizure was observed, videotaped behaviors were analyzed from the corresponding video recording. Behaviors associated with limbic seizures in rat typically included sudden cessation of activity, head bobbing, facial automatisms, prolonged immobility with staring, and alternating or bilateral clonus. SRS was defined when electrographic and behavioral correlates were present.

Figure 1.
Experimental schedule and hyperthermic seizure induction. Time table (**A**) shows an experimental schedule and numbers of the rats per assay. Representative figures (**B**) show the process of HT-induced seizures, which consist of arrest of hyperkinesia, followed by body flexion, and occasionally followed by clonic contractions of the limbs.
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Morphologic and molecular study

Groups of animals were sacrificed on days 1, 3, and 7 after the seizures for analysis of brain edema (n = 6 per group per time point), and on 14 and 60 days for analysis of cell death, cell birth, and microglia activation (n = 6 per group per time point). The entire brains of rats (normal control, n = 3; HT, n = 3; and HT + EPO, n = 3) during the acute and latent periods (D1, D7, and D60) were used for the gene expression array. The significant changes in gene expression on microarray for 60 days after febrile seizures were categorized into the following four patterns: continuously high (CH); continuously low (CL); rise and fall (RF); and going-up (GU). The CH or CL patterns in the HT group were defined when genes showed a >2-fold increase or decrease compared to the NT group at three time points. The RF pattern was defined when genes showed a >2-fold decrease following a >2-fold increase compared with the NT group, and the GU pattern was defined when the gene expression was increasing over time, compared with the NT group. In order to justify the array results, we performed reverse transcriptase (RT)-polymerase chain reaction (PCR) on several genes in the different categories with array samples and additional brain samples on days 1, 3, 7, 14, and 30 post-HT (n = 3 per group per time point). All animal experiments are detailed in Data S1.
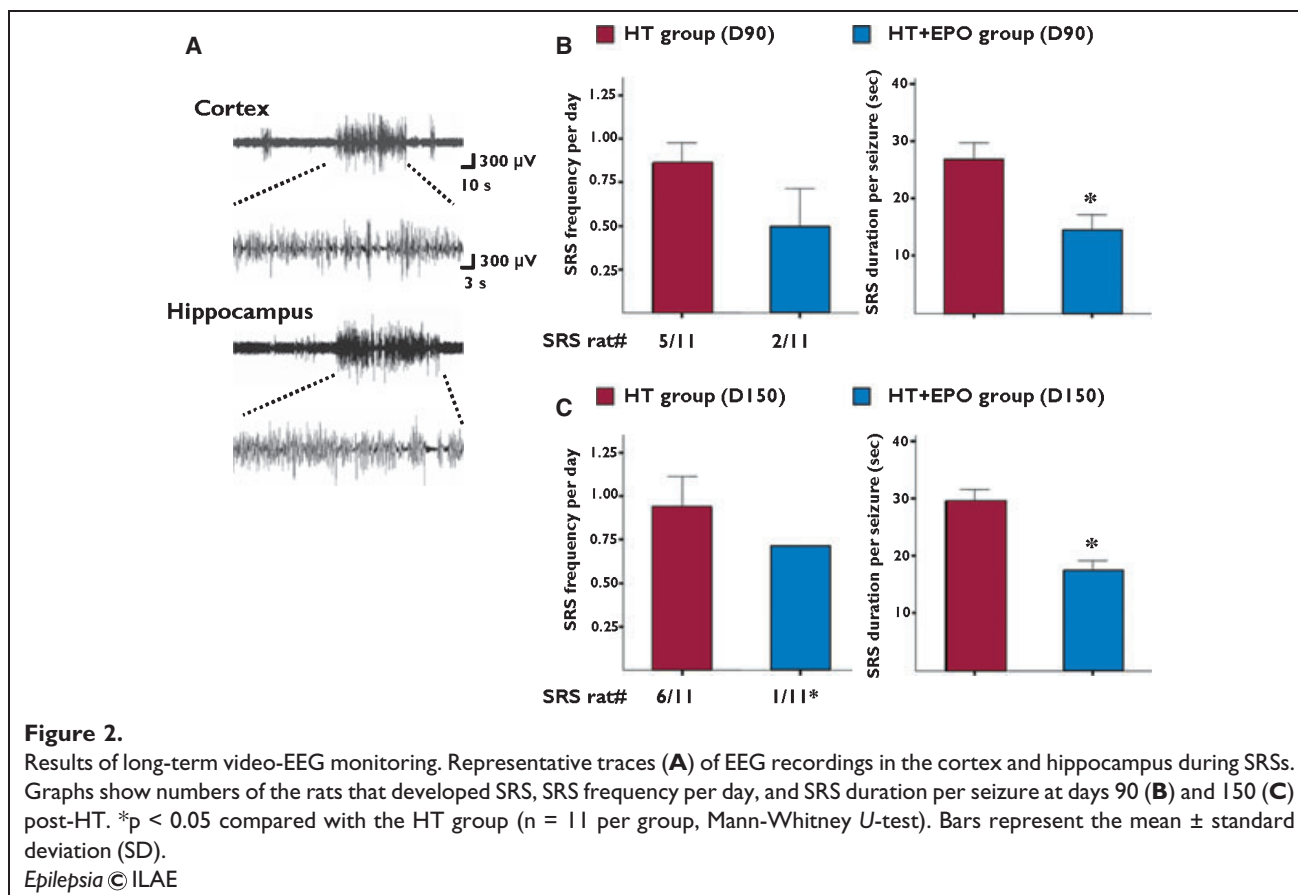
Results

Characteristics of HT-induced prolonged febrile seizures

The febrile seizures were characterized by arrest of hyperkinesias, followed by falling on their side or back with body flexion and clonic contractions of the limbs (Fig. 1B). This procedure resulted in stereotypical seizures in virtually all rats in both models of HT. There was little variation in the latency-to-onset of HT-induced seizures (3.2 ± 1.7 min). The mean duration of seizures was 21.7 ± 4.2 min in the 30RHT, and 38.5 ± 6.3 min in the 45HT. This protocol admitted most rats to survive for a long period (mortality rate: 45HT, 13.5%; 30RHT, 11.1%).

Reduction in the risk of SRS after febrile seizures by EPO

The same 11 rats per group were monitored for SRS in the two sessions (D90-D96 and D150-D156 post-HT). Greater than 300 h of video EEG per rat was available throughout the two sessions. Most of the seizures observed were generalized convulsive (stage 4/5) seizures, and focal seizures were observed only infrequently in some rats. Serial data from the two sessions showed a clear reduction of the SRS development by EPO. Electroclinical SRSs were

Figure 2.
Results of long-term video-EEG monitoring. Representative traces (**A**) of EEG recordings in the cortex and hippocampus during SRSs. Graphs show numbers of the rats that developed SRS, SRS frequency per day, and SRS duration per seizure at days 90 (**B**) and 150 (**C**) post-HT. *p < 0.05 compared with the HT group (n = 11 per group, Mann-Whitney *U*-test). Bars represent the mean ± standard deviation (SD).
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observed in 5 (45.5%) of 11 HT rats and in 2 (18.2%) of 11 HT + EPO rats (p = 0.170) in the early session, whereas in 6 (54.5%) of 11 HT rats and in 1 (0.09%) of HT + EPO rats (p = 0.022, chi square test). Although the seizure frequency in the rats that developed SRS was not statistically different between HT and HT + EPO groups (Fig. 2B,C; early session: p = 0.181; late session: p = 0.677, Mann-Whitney *U*-test), the seizure duration per event was significantly attenuated in the HT + EPO, compared to HT group (Fig. 2B,C; early session: 26.87 ± 11.1 s in the HT group, 14.50 ± 6.56 s in the HT + EPO group, p = 0.020; late session: 29.61 ± 7.90 s in the HT group, 17.33 ± 3.05 s in the HT + EPO group, p = 0.017, Mann-Whitney *U*-test). As one rat from HT + EPO group continued to develop 0.7 seizures per day throughout the two sessions (Fig. S1), the seizure frequency in the rats having SRS was not different between HT and HT + EPO groups. Except for this intractable rat, all HT + EPO rats underwent seizure cessation in the late phase.

No effect of febrile seizure on cell apoptosis or proliferation

The risk of epilepsy after a febrile seizure was attenuated by EPO. Given that cell death or genesis is required for epileptogenesis, we examined the neurodegenerative or neuroproliferative effects of HT on whole brains at 14 and 60 days after febrile seizure induction. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in the normal developing brains showed a small amount of labeled cells, and there were no significant differences in cell death among the NT, 45HT, 30RHT, and HT + EPO groups (Fig. S2). BrdU$^+$ cells were distributed throughout the developing brain, that is, neocortex, striatum, hippocampus, thalamus, and amygdala (Fig. S3A). A similar number of BrdU$^+$ cells was observed in 14-day brains of rats in the NT, 45HT, 30RHT, and HT + EPO groups (Fig. S3B). In addition, there was no difference in the proportion of cells with BrdU and NeuN or glial fibrillary acidic protein (GFAP) colocalization between the groups (Table S1).

## Modulation of febrile seizure-induced molecular alterations by EPO

To get a global view of the changes in gene expression in the brain undergoing febrile seizures, we performed serial gene expression arrays during the latent period (D1, D7, and D60 post-HT) prior to the onset of SRS. Approximately 35,000 genes were analyzed per experimental group (HT and HT + EPO). To delineate the epileptogenic process, we analyzed differentially upregulated and downregulated

genes of the HT and HT + EPO groups versus the normal and age-matched NT groups. There were small proportions of differentially regulated genes in the HT and HT + EPO groups compared to the NT group (Fig. S4). We tracked serial changes of individual genes for the latent period (D1, D7, and D60 post-HT), and categorized the genes into four types (Table S2). We detected 20 genes that were continuously high and 37 genes that were continuously low compared to the NT group. We also detected 337 genes with rising and falling patterns and 179 genes that were going up as time passed (Fig. 3). The potential target genes were assigned by comparing the levels with those of the HT + EPO group; therefore, the ontology of genes regulated differentially after febrile seizures was determined. The CH genes appeared to be associated primarily with cell cycle and adhesion. The expression of many genes related to energy metabolism was CL. Within the category of RF, the largest changes were for genes involved in inflammation, apoptosis, and GABA signaling. The GU category included genes involved in ion transport and synaptogenesis (Fig. 3). By using RT-PCR, we could confirm the microarray results that showed the selective changes of genes in the RF and GU category at the different time points and in HT and HT-EPO group (Fig. S5).

Upregulation of inflammatory cytokines and cystatin C by febrile seizures

RT-PCR was performed on a set of differentially regulated genes of the RF group (TNF-α, IL-6, and IL-1β). Rats were sacrificed on days 1, 3, 7, 14, and 30 after hyperthermic seizures, and normal age-matched rats served as controls. TNF-α, IL-6, and IL-1β were upregulated in the brains during the early period immediately after HT by comparison to the samples from the NT group (Fig. S6A). The magnitude of change detected between the two groups was comparable to microarray data. Optical density results confirmed the effect of HT on inflammatory cytokine gene expression in the immature rat brain (Fig. S6B; $p < 0.05$, $n = 3$ per group, Mann-Whitney U-test). Because cystatin C has been linked to epilepsy (Hendriksen et al., 2001) and the expression of cystatin C is greatly increased in gene expression array, we hypothesized that cystatin C participates in the cellular alterations that underlie network reorganization during epileptogenesis after febrile seizures. The time course of cystatin C protein expression in the rat brain was investigated 1, 3, 7, 14, and 30 days after the induction of febrile seizures. Western blot analysis revealed that cystatin C protein expression was increased on day 1, peaked between days 3 and 14, and remained high for 30 days
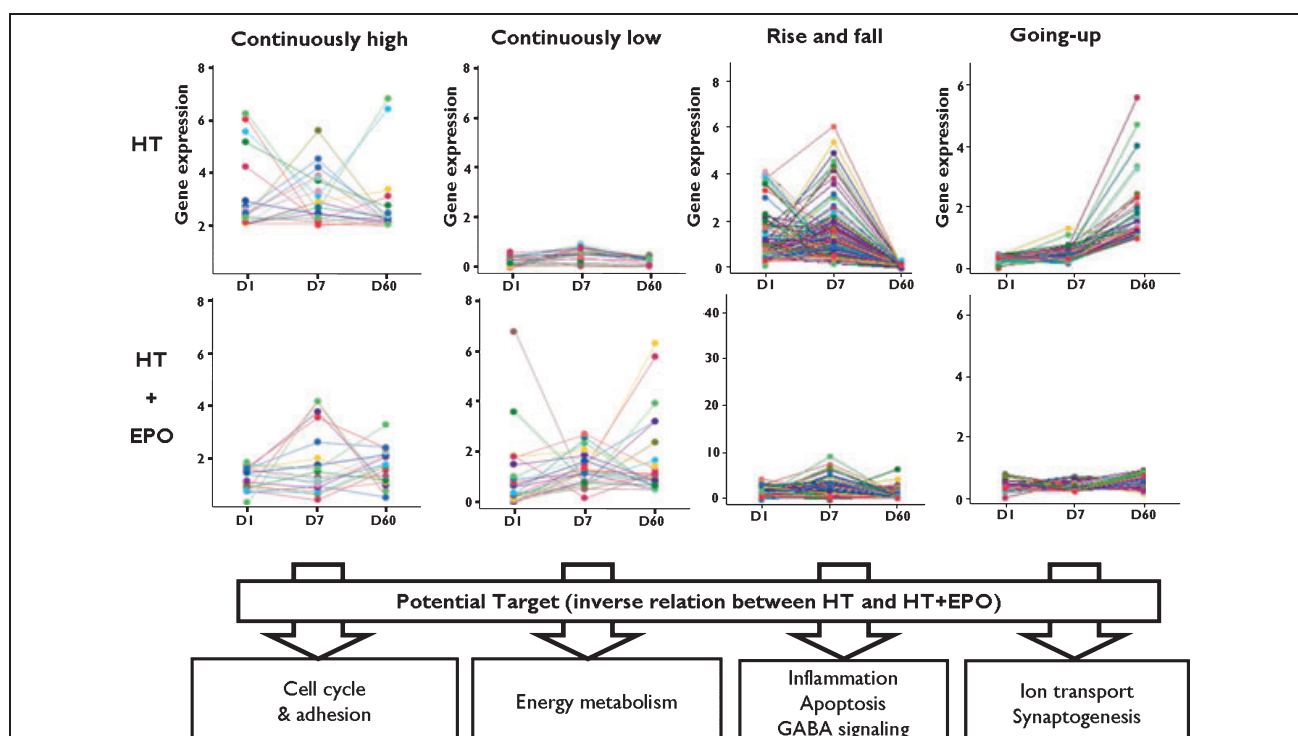
Figure 3.
Serial changes of potential target genes. Graphs depict genes that are continuously high, continuously low, rising and falling, and going-up during the epileptogenic period after febrile seizures. Time points for array are plotted on the x axis, and the expression intensities of genes in the HT and HT + EPO groups in comparison to the NT group are plotted on the y axis. Boxes provide potential target genes that show an inverse relationship between the HT and HT + EPO groups.
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after febrile seizures (Fig. S6; p < 0.05, n = 4 per group, Mann-Whitney *U*-test).

Intervention of early inflammatory changes following febrile seizures by EPO

Given the increase in inflammatory responses and cystatin C signaling after febrile seizures described earlier, we investigated whether the administration of EPO would modulate the early molecular changes in the brain. EPOR mRNA was slightly detectable in control tissues, but dramatically elevated in epileptic brains 1–7 days post febrile seizure, with an apparent peak observed on day 1 (Fig. 4A,B; p < 0.05; n = 3, Mann-Whitney *U*-test). In the HT + EPO group, the increase in the protein expression of cystatin C and the mRNA expression of TNF-α, IL-6, and IL-1β were significantly attenuated from day 1 after febrile seizures, as compared with the HT group (Fig. 4C,D; p < 0.05, n = 3 per group, Mann-Whitney *U*-test). Next, we measured the brain water content during the early phase after febrile seizures, because it is the most important BBB breakdown-related response (Xi et al., 2006). The Mann-
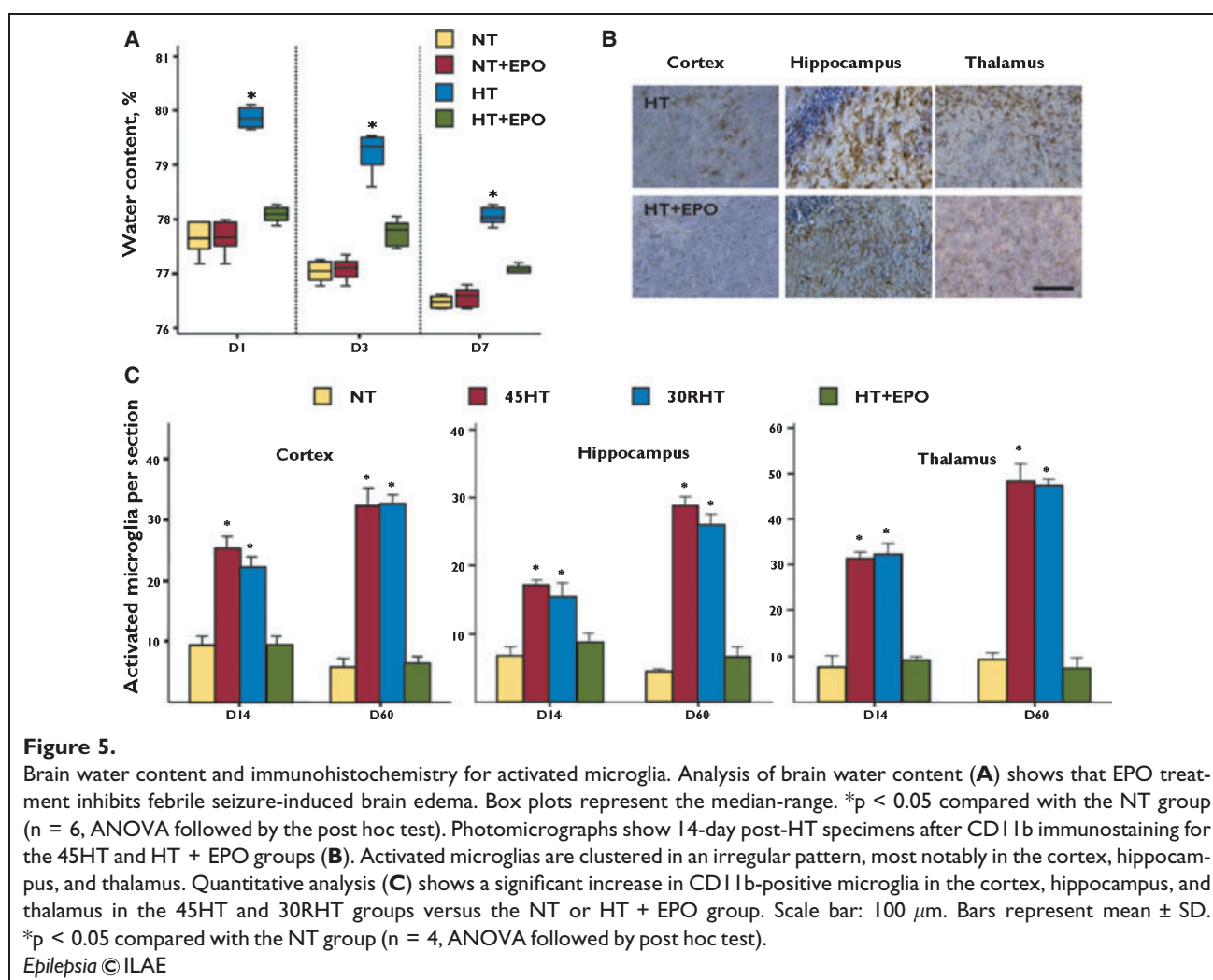
Whitney *U*-test suggested an intergroup difference among the brain water content of the HT and NT groups over the course of 7 days (Fig. 5A). HT significantly increased the mean brain water content as compared to NT. When brain water content was also measured in the HT + EPO group over the course of 7 days, EPO significantly reduced the mean brain water content after febrile seizures [Fig. 5A; p < 0.05; n = 6, analysis of variance (ANOVA) followed by the post hoc test]. Neuroinflammation was evaluated by immunohistochemical staining for CD11b. NT rats showed homogeneous distributions of cells with long, fine processes extending from small cell bodies. In rats undergoing febrile seizures, activated microglial cells immunostained with CD11b with a few short processes and large cell bodies were observed in the cortex, hippocampus, and thalamus in an irregular pattern 14 days after febrile seizure induction (Fig. 5B). EPO treatment reduced the microglial response after febrile seizures. Quantification analysis (Fig. 5C) revealed that the rats in the 45HT and 30RHT groups had more activated microglial cells than those in the NT group at 14 days. The number of activated microglia in the two
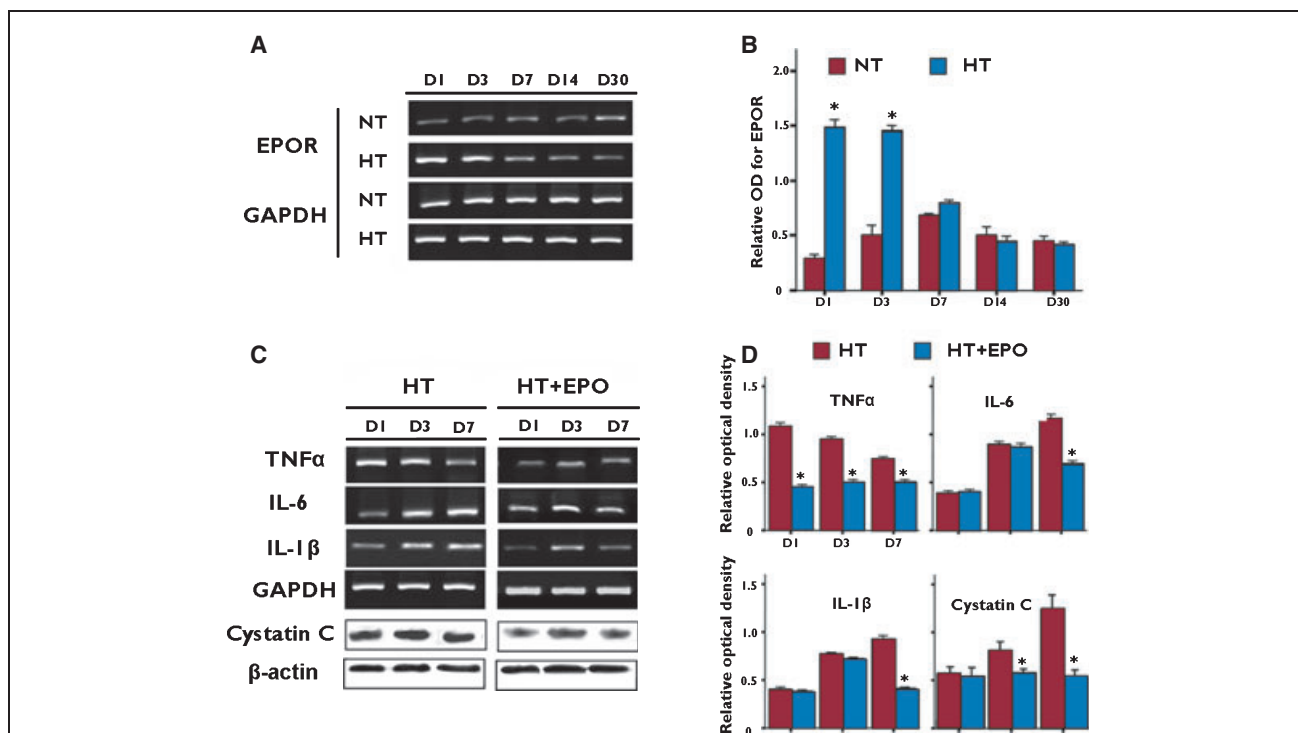
Figure 4.
Changes of early molecular signals by RT-PCR and western blot. RT-PCR (**A**) documents the time course of EPOR in the NT, HT, and HT + EPO groups. Results are expressed as optical density ratios versus glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Febrile seizures caused EPOR gene upregulation from day 1 post-HT and remained high until day 7 (**B**). RT-PCR and western blotting document the time course of TNF-α, IL-6, and IL-1β mRNA and cystatin C protein in the HT and HT + EPO groups (**C**). Results are expressed as optical density ratios versus GAPDH or β actin. Densitometric analysis (**D**) of the band indicated a significant decrease in the expression of TNF-α, IL-6, and IL-1β mRNA in the HT + EPO group, as compared with the HT group. Bars represent mean ± SD. *p < 0.05 compared with the HT group (n = 3 per group, Mann-Whitney *U*-test).
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Figure 5.
Brain water content and immunohistochemistry for activated microglia. Analysis of brain water content (**A**) shows that EPO treatment inhibits febrile seizure-induced brain edema. Box plots represent the median-range. *p < 0.05 compared with the NT group (n = 6, ANOVA followed by the post hoc test). Photomicrographs show 14-day post-HT specimens after CD11b immunostaining for the 45HT and HT + EPO groups (**B**). Activated microglias are clustered in an irregular pattern, most notably in the cortex, hippocampus, and thalamus. Quantitative analysis (**C**) shows a significant increase in CD11b-positive microglia in the cortex, hippocampus, and thalamus in the 45HT and 30RHT groups versus the NT or HT + EPO group. Scale bar: 100 μm. Bars represent mean ± SD. *p < 0.05 compared with the NT group (n = 4, ANOVA followed by post hoc test).
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HT groups remained higher than the NT group 60 days after febrile seizures. Remarkably, there was a significant decrease in the CD11b-positive microglia in the cortex, hippocampus, and thalamus in the HT + EPO group 14 and 60 days after febrile seizures as compared to the 45HT or 30RHT groups (p < 0.01, n = 6 for each group, ANOVA followed by post hoc testing).

DISCUSSION

Prolonged experimental febrile seizures lead to later epilepsy. We identified expression changes for genes that may encode key factors involved in epileptogenesis after febrile seizures. EPO treatment immediately after febrile seizures could attenuate the risk of chronic behavioral seizures by modulation of early key molecules that govern the inflammatory response and neuronal network function.

Febrile seizures are the most common type of seizure in children, affecting 3–5% of young children (Hauser, 1994). It is generally believed that simple febrile seizures are benign and do not predispose to later neurologic

pathologies, but complex febrile seizures (focal, prolonged, or recurrent within 24 h) are associated with an increased risk for epilepsy. We attempted to develop febrile seizure models with more obvious changes to intervene the epileptogenic process. We utilized a simple yet reproducible 45HT model, which showed little association with immediate morbidity, thereby permitting prospective long-term analyses of the effects of febrile seizures on the development of later epilepsy. The 45HT model could induce recurrent, spontaneous, electroclinical seizures later in life in 50% of rats, and, therefore, it represents a useful model for studying the relationship between febrile seizures and epilepsy.

A classical mechanism of epileptogenesis in the mature hippocampus involves loss of vulnerable populations of neurons and reorganization of the remaining circuit. In experimental febrile seizure, the cell death does not accompany the epileptogenic process, indicating that cell death is not required for the generation of epilepsy (Toth et al., 1998; Bender et al., 2004; Dubé et al., 2006). Our data confirm these observations in the model of prolonged febrile

seizures, or even repeated prolonged seizures. Aberrant cell genesis is another hypothesis that has recently attracted much attention to epileptogenesis research. However, there are no significant changes in the number of surviving newborn cells after febrile seizures (Bender et al., 2004; Lemmens et al., 2008). Similar observations made in the present study suggest that epileptogenic processes initiated early in development may depend on remodeling of pre-existing cells or circuit, rather than cell death or birth.

Remodeling of neuronal circuits leading to later epilepsy can be associated with altered gene expression (Chen et al., 1999, 2001; Dubé et al., 2000). Several genes have been implicated in epileptogenesis following febrile seizures, including those encoding ion channels (Brewster et al., 2002), endocannabinoid receptors (Chen et al., 2003), and GABA$_A$ receptors (Tsai & Leung, 2006). We analyzed the expression of >30,000 genes during the serial latent periods to delineate long-lasting circuitry remodeling. As the experimental febrile seizure perturbs neuronal activity in cortical and limbic circuits without definite hippocampal pathology (Dubé et al., 2006), we utilized the entire brain sample for array. We pooled RNA by combining three RNA samples from each group to reduce the influence of intersample variability, and then checked RT-PCR in array samples and additional brain samples (n = 3 per time per group) to see whether the significant changes on microarray [e.g., continuously high (CH); continuously low (CL); rise and fall (RF); and going-up (GU)] would be a real phenomenon. PCR data might not indicate an epileptogenic process because some of the rats would not develop seizures. Given that the interictal epileptiform discharges were recorded in 88.2% of rats that underwent 30-min hyperthermia (Dubé et al., 2006), we speculated that the molecular changes would occur without overt seizures during the short monitoring periods.

Our microarray analysis of brains undergoing prolonged febrile seizures identified expression changes for genes involved in inflammation, synaptogenesis, synaptic transmission, and metabolism, several of which have been implicated as effectors of epileptogenesis. Proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, can influence neuronal function and excitability in a variety of epilepsy models (Vezzani et al., 1999; De Simoni et al., 2000; Dubé et al., 2005; Vezzani & Granata, 2005). Human studies have revealed increased plasma and cerebrospinal fluid levels of some inflammatory cytokines in patients with febrile seizures and intractable epilepsy (Virta et al., 2002; Lehtimäki et al., 2004). The present study showed that TNF-α, IL-6, and IL-1β expression increased from day 1 and remained high on day 7. Febrile seizure-induced early inflammatory responses could have a lasting effect on the constitution of the hippocampal neuronal network, which may contribute to the development of a hyperexcitable state. However, it remains to be clarified whether either hyperthermia itself or hyperthermic seizures result in the significant molecular changes because we did not have a control group of hyperthermia without seizures.

Some additional pathways identified by our transcriptome analysis may also be significant during epileptogenesis. One such gene is cystatin C, a cysteine proteinase inhibitor. The expression of cystatin C is increased in a delayed manner from 4–7 days after SE in both rats and mice, and it is also prominent during the chronic phase of epilepsy both in a rat model of TLE and in human patients with TLE (Aronica et al., 2001; Hendriksen et al., 2001; Lukasiuk et al., 2003). However, whether cystatin C underlies the development of a lowered seizure threshold and subsequent epilepsy remains unknown. Although cystatin C from astrocytes may protect neurite growth from degradation by cysteine proteases and thus facilitate regeneration (Ying et al., 2002), there are also reports suggesting that, rather than being a protective factor, cystatin C is detrimental to the brain and functions as a mediator of cell damage (Nagai et al., 2002). Our findings suggest that febrile seizures occurring early in life could program the epileptogenic process through bifunctional cystatin C activity.

Normal neuronal activity requires a stable extracellular environment maintained by proper functioning of the BBB. Vasoactive, proteolytic, and chemotactic factors and proinflammatory cytokines might trigger acute BBB permeability changes and modulate neuronal targets. BBB disruption has been shown both in epileptic rats and humans, both shortly after SE and during the chronic epileptic phase (Ilbay et al., 2003; Leroy et al., 2003; Uzum et al., 2006; van Vliet et al., 2007). Although it is not known how alterations in BBB permeability contribute to SRS following febrile seizures, it is possible that BBB opening and cellular exposure to serum-derived brain toxic components initiate an inflammatory response that eventually leads to epileptiform activity. Microglial activation can also influence neuronal function via the so-called bystander effect, which seems to be caused by the release of a combination of neurotoxic substances, such as glutamate and nitric oxide (NO), and of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α (Vezzani et al., 1999; De Simoni et al., 2000; Rizzi et al., 2003; Jung et al., 2006; Chu et al., 2008). In our model, BBB breakdown was induced immediately after febrile seizure, and it was associated with microglial activation in limbic structures, the neocortex, and the thalamus for at least 60 days after febrile seizures.

Although the mechanisms that may interact to render the immature brain epileptic remain elusive, the transcriptional profiles in the present study provide the hypothesis that epileptogenesis is tailored by the early inflammatory responses. This hypothesis was strongly supported by the outcome of the experiments conducted using EPO, a pleiotropic molecule. EPO has been reported to reduce the infiltration of macrophages, production of inflammatory cytokines, microglial activation, and BBB breakdown in a variety of epilepsy models (Brines et al., 2000; Yatsiv et al.,

2005; Savino et al., 2006; Uzum et al., 2006; Nadam et al., 2007; Villa et al., 2007; Chu et al., 2008). Therefore, EPO is attracting interest as a possible antiepileptogenic agent after prolonged febrile seizures. In the present study, EPO receptors increased from the early period after febrile seizures and EPO treatment reduced the risk of epilepsy by modulating the early alterations in the expression of multiple genes, including inflammation, and suppressing BBB breakdown and microglial activation.

Our findings suggest that EPO treatment may provide a new strategy for preventing epilepsy in susceptible individuals undergoing complex febrile seizures. However, since the 45-min hyperthermia model applied in this study may be peculiar considering the epilepsy rate is much higher than in humans, the interventional strategy in children has to be cautiously considered.

Acknowledgment

Disclosure

The authors declare no conflicts of interest. 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.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and Methods.

Figure S1. Individual data of long-term video-EEG monitoring.

Figure S2. Histologic analysis of cell apoptosis.

Figure S3. Histologic analysis of cell proliferation.

Figure S4. Transcriptional profiles of the brains undergoing febrile seizure.

Figure S5. Validation of genes in the specific category on microarray.

Figure S6. Early changes in the expressions of inflammatory cytokine genes following febrile seizures.

Table S1. Cell genesis, survival and differentiation in brain regions postfebrile seizure.

Table S2. Differentially regulated gene profiles (Excel sheet).

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