Antagonism of peripheral inflammation reduces the severity of status epilepticus

Nicola Marchi a,b, Qingyuan Fan a,b, Chaitali Ghosh a,b, Vincent Fazio a,b, Francesca Bertolini b, Giulia Betto a,b, Ayush Batra a, Erin Carlton a,b, Imad Najm d, Tiziana Granata e, Damir Janigro a,b,c,*

a Cerebrovascular Research, Cleveland Clinic Lerner College of Medicine, Cleveland, OH 44106, USA
b Department of Neurology and Cell Biology, Cleveland Clinic Lerner College of Medicine, Cleveland, OH 44106, USA
c Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, Cleveland, OH 44106, USA
d Department of Neurology, Cleveland Clinic Lerner College of Medicine, Cleveland, OH 44106, USA
e Istituto Neurologico Besta, Milano, Italy

ARTICLE INFO

Article history:
Received 15 July 2008
Revised 30 September 2008
Accepted 3 October 2008
Available online 28 October 2008

Keywords:
Blood-brain barrier
Cerebrovascular function
Epilepsy
Inflammation
Anti-inflammatory therapy
New drug targets
T lymphocytes

ABSTRACT

Status epilepticus (SE) is one of the most serious manifestations of epilepsy. Systemic inflammation and damage of blood-brain barrier (BBB) are etiologic cofactors in the pathogenesis of pilocarpine SE while acute osmotic disruption of the BBB is sufficient to elicit seizures. Whether an inflammatory-vascular-BBB mechanism could apply to the lithium–pilocarpine model is unknown. LiCl facilitated seizures induced by low-dose pilocarpine by activation of circulating T-lymphocytes and mononuclear cells. Serum IL-1β levels increased and BBB damage occurred concurrently to increased theta EEG activity. These events occurred prior to SE induced by cholinergic exposure. SE was elicited by lithium and pilocarpine irrespective of their sequence of administration supporting a common pathogenetic mechanism. Since IL-1β is an etiologic trigger for BBB breakdown and its serum elevation occurs before onset of SE early after LiCl and pilocarpine injections, we tested the hypothesis that intravenous administration of IL-1 receptor antagonists (IL-1ra) may prevent pilocarpine-induced seizures. Animals pre-treated with IL-1ra exhibited significant reduction of SE onset and of BBB damage. Our data support the concept of targeting systemic inflammation and BBB for the prevention of status epilepticus.

© 2008 Published by Elsevier Inc.

Introduction

In spite of the advancements in therapeutic interventions for seizure disorders, 102,000 to 152,000 individuals are affected each year in the United States (with 126,000 to 195,000 status epilepticus episodes), and over 22,000 to 42,000 deaths per year occur in patients with status epilepticus (SE) (Shorvon et al., 2008). There is increasing evidence that systemic inflammation may have significant effects of normal brain function and may in particular lead to epileptic seizures and SE (Vezzani and Granata, 2005; Marchi et al., 2007b; Marchi et al., 2007a; Uva et al., 2008; Galic et al., 2008). For example, fever is the most common correlate of immune activation against pathogens, and hyperthermia has been for a long time recognized as a powerful trigger for seizures. More recently, clinical (Elkassabany et al., 2008; Marchi et al., 2007a; Korn et al., 2005) and translational (Korn et al., 2005; Tomkins et al., 2007; Ivens et al., 2007) studies have shown that the gatekeeper at the brain-systemic circulation interface, the blood-brain barrier, may be a crucial etiological player in epilepsy (Ohy and Janigro, 2006). These concepts have been expanded to a popular model of limbic epilepsy, namely the pilocarpine model (Marchi et al., 2007b; Uva et al., 2008). Pilocarpine caused acute peripheral pro-inflammatory changes leading to blood-brain barrier (BBB) leakage prior to SE (Marchi et al., 2007b). These studies also demonstrated that it is unlikely that cholinergic drugs such as pilocarpine may act directly on neurons since pilocarpine was relatively impermeant across the BBB. Consistent with this hypothesis is the fact that when directly applied to the brain, pilocarpine failed to produce electrographic seizures (Marchi et al., 2007b; Uva et al., 2008). While converging evidence points to the BBB failure as a trigger for limbic seizures and cholinergic SE, it is not clear whether these transient episodes of BBB dysfunction are truly necessary for seizure development. This is particularly intriguing in the muscarinic models of SE where pilocarpine exerts two independent effects, one aimed at BBB integrity (Marchi et al., 2007b) and one promoting gamma oscillations in the hippocampus and related structures (Uva et al., 2008). Furthermore, it is not currently known if induction of BBB leakage by non-cholinergic means prior to pilocarpine exposure is equally effective in facilitating the latter epileptogenic actions of the drug.

To test the effect of a pro-inflammatory stimulus prior to administration of seizure promoting agents we used the lithium–pilocarpine...
model, where pretreatment with the psychoactive ion allows to reduce the minimal epileptogenic dosage of pilocarpine to 1/10th of the dosage required in absence of pretreatment (Cavalheiro et al., 2006). Lithium salt compounds are widely employed in the treatment of manic-depressive psychosis. Patients receiving lithium therapy often demonstrate an unexplained increase in white blood cell counts, with granulocytosis being the most common finding (Carmen et al., 1993). Pronounced EEG changes were also reported (Helmchen and Kanowski, 1971).

In the presented study we tested the hypothesis that increased peripheral immunological mediators and blood-brain barrier disruption are mechanism of lithium’s pro-epileptogenic effects. We also tested the efficacy of anti-inflammatory molecules in reducing the onset SE induced by cholinergic activation.

Methods

EEG recording and video monitoring

Stereotactic electrode implantation was performed under pentobarbital anesthesia (45 mg/kg i.p.), using the Kopf stereotactic frame and a stereotactic atlas of the rat brain. For intrahippocampal recordings, bipolar twisted stainless wire electrodes (0.1 mm diameter; 0.5 mm vertical tip separation, Medwire, New York, New York, USA) are placed bilaterally in the dorsal hippocampus (LH: left hippocampus, RH: right hippocampus, A: $-3.9$ mm, L:±3.0 mm, D: $-2.5$ mm from Bregma). Stainless steel screws (MX-0090-2, Small Parts Inc., Miami, Florida) were placed bilaterally on the dura mater of the frontal cortex (LC: left frontal cortex, RC: right frontal cortex, A: 1.0 mm, L:±2.5 mm from Bregma). An additional screw electrode is placed in the frontal sinus and as a referential recording electrode. Rats were left unrestrained for 2 weeks for recovery from surgery before EEG recordings are performed. Each rat was kept in separate cage, under 12-hour dark-light cycles, with free access to food and water. Digital EEG recordings (5 channels per rat) were performed using two Vanguard systems (Lamont, Madison, WI, USA). Each system consists of a recording Hewlett-Packard workstation and a similar review system connected to two 21-inch monitors. EEG data were sampled a rate of 100 Hz. Spectrum and spike amplitude analysis was performed at time shown in Fig. 1B. We also used the Pinnacle Technologies EEG-Video recording apparatus for 24/7 monitoring. Comparison of results did not reveal any significant quantitative changes but this new and more compact system has several practical advantages over the Vanguard apparatus. The model we use, 8206, does not require any additional acquisition cards or amplifiers. All data are transferred via a USB connection to a PC. The USB connection also provides power to the 8206 and preamplifier. Origin FFT software is used in conjunction to the acquisition system for data analysis.

Animals and tissue sample preparation

Rats were housed in a controlled environment (21 ± 1 °C; humidity 60%; lights on 08:00 AM–8:00 PM; food and water available ad libitum). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec.12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Rats (male Sprague–Dawley 200–250 g) were injected with: 1) LiCl 3meq/kg followed 20 h later by methylscopolamine (1 mg/kg, i.p., Sigma-Aldrich) and pilocarpine (30 mg/kg); 2) methylscopolamine (1 mg/kg, i.p.) and pilocarpine (30 mg/kg) followed 1 h later by LiCl 3meq/kg; 3) methylscopolamine (1 mg/kg, i.p., Sigma-Aldrich) 30 min before pilocarpine (320 mg/kg i.p.); 4) Rat IL-1α (R&D System) was administrated in the tail vein (3.5–35 μg/kg) 2 h before scopolamine (1 mg/kg) and pilocarpine (320 mg/kg) treatment. See also Fig. 1A for number of rats used.

Animals were anesthetized with isoflurane at the following time points: 1) three and 20 h after LiCl injection; 2) during recurrent motor seizures and at onset of status epilepticus (SE) as assessed by behavioral observation (stages 4 and 5 of Racine’s scale) and EEG recordings; 3) 6 h after SE onset. Blood for FACS analysis was withdrawn from the right heart ventricles; brain areas (hippocampus and entorhinal cortex) were resected and frozen. Serum samples for ELISA testing were obtained after centrifugation at 1500 g for 10 min at 4 °C. See Fig. 1B.
**FACS and cytokine ELISA**

For FACS analysis, 150 μL of whole blood (or brain homogenized) were incubated with a combination of specific antibodies recognizing different population of WBC. In particular mouse anti-rat CD8α-FITC, CD3-R-PE and CD4-PE-Cy5 (BD-Pharmingen) were added (2 μL, 5 μL and 5 μL respectively) to blood samples. Mouse anti-rat CD11b-FITC and CD3-R-PE were added (2 μL, and 5 μL respectively) for the determination of neutrophils/monocytes. Blood samples were analyzed by the FACS-Core facility at LRI. IL-1β ELISA tests were purchased from Pierce Biotechnology Inc and performed as described by the vendor.

**FITC-albumin solution composition**

A recently developed microangiographic technique based on the injection of fluorescent probes enables confocal visualization of the entire brain micro- and macrovasculature (Cavaglia et al., 2001). Simultaneous observation of functional changes in BBB permeability is also possible if appropriate microangiographic molecules are employed. To this end, fluorescent albumin was used, since albumin does not extravasate abuminally if the BBB is intact (Cavaglia et al., 2001). The fluorescent albumin solution was prepared by reconstituting 500 mg of bovine desiccate albumin-fluorescein isothiocyanate (FITC-albumin, MW 69 KD; 12 mol/mol albumin; Sigma, St. Louis, MO) in 50 mL of phosphate buffered saline (0.1M PBS) lacking magnesium and calcium ions. The FITC solution was stirred at room temperature in the dark for 5 min prior to injection. FITC albumin was directly infused in the left ventricle (Cavaglia et al., 2001). 10 mL of solution were injected at rate of 1 mL/min under general anesthesia. Micro-angiography was performed 3 and 20 h after LiCl treatment (see Fig. 1B). Presence of vascular leakage in treated and control animals was evaluated by visual inspection and by confocal-microscopy (see also below).

![Fig. 2.](image-url) Electrophysiological effects of cholinergic drugs and lithium in the rat brain. (A, B) show baseline activity and normal activity following exposure to sub-convulsive dosage of pilocarpine (30 mg/kg). Low dosage of pilocarpine never led to electrographic changes consistent with seizure-like activity, but frequently were associated with synchronous (hippocampal–cortical, left–right) clusters of activity indicated by black asterisks. Exposure to lithium caused subtle changes after 3 h and a more pronounced synchronized activity at 20 h post exposure (C). At 20 h after lithium, the EEG signals resembled the outcome of sub-threshold pilocarpine, but both synchronous (black asterisks) and asynchronous, unilateral spikes were seen; the latter are indicated by gray asterisks. When lithium-induced EEG changes became most apparent, a sub-convulsive dosage of pilocarpine was administered (D). The combined effect of pre-exposure to lithium and sub-convulsive pilocarpine lead to the development of tonic chronic seizures leading to status epilepticus. Reversal of the administration schedule (i.e., pilocarpine at sub-convulsive dosage followed by lithium, see Fig. 1A) gave an almost identical progression towards SE (E).
Quantitative measurement of Evan's blue dye by fluorescent analysis

For the quantification of brain Evan's blue we used: 1) control rats; 2) pilocarpine-treated rats (320 mg/kg) sacrificed at onset of status epilepticus as observed behaviorally (tonic–clonic convulsion corresponding to stage 5 of the Racine Scale); 3) rats pre-treated with 35 μg/kg of IL-1ra 2 h prior injection of 320 mg/kg of pilocarpine. Rats were perfused with Evan's Blue solution (4 ml/kg, 2% in PBS) through the left ventricle. After 2–3 min rats were perfused with 20 ml of PBS (5 ml/min) in order to wash out the intravascular volume of Evan’s Blue. Rats were then decapitated, brain areas dissected out and frozen. Frozen tissue sections were weighed and placed in 50% trichloroacetic acid (TCA) solution for homogenization in tapered 1.5 mL Eppendorf sample tubes using a Teflon coated pestle. The homogenate from each sample was then centrifuged at 8000 rpm in a micro centrifuge for 10 min; the clear supernatant was removed and diluted 1:3 with 95% ethanol. If a hue of blue dye was visible in the supernatant, the extract was diluted an additional 10-fold in a diluent matrix or 1:3 50% TCA: 95% ethanol. The fluorescent signal was measured using an excitation wavelength of 530 nm (25 nm bandwidth) and an emission wavelength of 645 nm (bandwidth 40 nm) on a BioTek Synergy HT Microplate reader (Winooski, VT). The fluorescence signal was converted to a concentration of Evan’s Blue dye by comparing the signal to that of prepared standards ranging from 0 to 500 ng/mL. The signal was linear throughout the range of dye from 100 to 500 ng/mL. Samples and standards were analyzed in triplicate. Final concentrations of Evan’s Blue dye were reported in micrograms of dye per gram of tissue.

Statistical analysis was performed with aid of Origin 7.0 (Microcal) and Jump 7.0 (www.jump.com); data were considered to be significantly different when \( p < 0.05 \) (by ANOVA).

**Fig. 3.** Effects of lithium on EEG. Abnormal, but not “epileptic” EEG recordings were acquired. (A) Power spectral analysis of 25 min of continuous intracortical recordings obtained prior to lithium exposure (black trace) and 3 h after administration of 3 mEq/kg (red trace). Note that a pronounced increase in 4–5 Hz activity was observed after LiCl exposure. LC = left cortex, RC = right cortex. The asterisks in A1 show the time interval used in B to reveal the sharp increase in a specific frequency range corresponding to theta activity. The segment was chosen based on prolonged (hours) recordings. (C) In addition to a specific appearance of increased theta activity, the amplitude and number of individual events occurring at 4 to 5 Hz was also dramatically increased. Data analysis was performed with Origin software (Microcal, v 7.0).
Fig. 4. Exposure to lithium causes a rapid permeability increase of the BBB. Blood brain barrier (BBB) integrity was assessed by intra-arterial perfusion of FITC-labeled albumin 3 and 20 h after LiCl injection. This technique allows the visualization of the cerebrovasculature and reveals eventual leaks across the BBB (Cavaglia et al., 2001). In other words, this technique is a variation of microangiography where instead of contrast agents a fluorescent label (in this case FITC-albumin) is injected intrarterial prior to sacrifice and fixation. Some sections were counterstained for the astrocytic marker GFAP or for the nuclear stain DAPI (B). Note that in naive animals (A) vascular integrity and capillary networks were evident and homogeneously distributed in cortical sections (A). In animals pretreated with lithium (B) abundant yet circumscribed leakage of the blood-brain barrier was observed 3 h after LiCl injection. Note that the extravasation of the fluorescently-labeled albumin resulted in depletion of the marker that normally allows visualization of the entire vascular structure. Thus, upstream from leaky spots the filling of the vasculature is less pronounced (indicated by white asterisks). The changes in blood-brain barrier permeability induced by lithium were predominantly seen in frontal and temporal cortex, including hippocampus and surrounding structures (arrows in C-F, relative to 20 h after LiCl injection). D shows an enlargement of the region in C highlighted by a dotted line. E refers to another region in the controlateral cortex to emphasize leakage at the level of presumed capillary structures. Widespread leakage in temporal cortex is shown in F. Occasional leaks were also observed in the thalamus (G).
Results

Seizures induced by pilocarpine or lithium–pilocarpine were staged by the methods developed by Racine and modified by Gilbert (Bawden and Racine, 1979; McIntyre and Racine, 1986; Gilbert, 1995). For the experiments described herein, staging of limbic seizures was performed by video EEG monitoring. Fig. 1A, B summarizes the treatments used in our experiments. We exposed rats to saline treatment, to sub-convulsive or convulsive levels of pilocarpine (30 and 350 mg/kg respectively), or to lithium chloride (3 mEq/L). Fig. 2 shows typical EEG patterns recorded under control conditions (A), after exposure to sub-convulsive pilocarpine levels (B), or after exposure to lithium (C, D). Note the normal EEG appearance after low pilocarpine exposure. While no frank behavioral seizures were observed three to 20 h after exposure to lithium, the EEG progressively synchronized to a burst firing pattern. Increased theta activity (4–5 Hz) and spike amplitude was observed starting from 3 to 4 h after LiCl treatment (Fig. 3). This is comparable to the acute effects of lithium in patients where a decreased seizure threshold or SE has been reported at therapeutic concentrations of the drug (Kuruvilla and Alexander, 2001; Roccatagliata et al., 2002; Addy et al., 1986). As expected, when sub-convulsive levels of pilocarpine were administered 20 h after exposure to lithium, behavioral and electrographic seizures were observed (Fig. 2E). The same results were obtained when the treatment schedule was reversed. In fact, when we exposed the animals to sub-convulsive pilocarpine followed (1 h) by lithium, an initiation of seizures similar to that observed in the traditional lithium pilocarpine model was seen (Fig. 2E). These seizures progressed to full blown SE as seen in the traditional lithium–pilocarpine and high-dosage pilocarpine models. The selective permeability of the BBB after exposure to lithium was determined by intrarterial fluorescent techniques (Marchi et al., 2007b) to demonstrate frequent focal leakage of the cerebral vasculature three and 20 h after exposure to lithium (Fig. 4). These changes were predominantly evident in the limbic system and neocortex. Several other abnormalities were observed hours after exposure to lithium (Fig. 5). For example, ventricular enlargements and ventricular asymmetry were common in proximity to periventricular FITC albumin leakage and reactive gliosis.

Since therapeutic exposure to lithium is known to affect white blood cell counts in patients (Carmen et al., 1993), we tested its effects in rats by measuring monocyte and lymphocyte activation three and 20 h after exposure. The data in Fig. 6A–C demonstrate pronounced peripheral pro-inflammatory changes triggered by LiCl; these were characterized by an increased percent of CD3-CD11+ cells and decreased CD4:CD8 ratios. Albeit much less pronounced, similar trend were seen at sub-convulsive dosage (30 mg/kg) of pilocarpine (data not shown). Since one of the common inflammatory mediators involved in BBB dysfunction is IL-1β, we also tested cytokine serum levels in animals exposed to lithium. Our findings demonstrate a sudden increase of IL-1β serum levels following lithium (Fig. 6D). These changes were of similar magnitude as those seen after injection of convulsive dosages of pilocarpine (see also Marchi et al., 2007b). Note that IL-1β levels measured after SE were significantly increased.

Fig. 5. The effects of lithium are not limited to cerebrovascular permeability but extend to altered CSF circulation and periventricular gliosis. After exposure to lithium, animals presented with asymmetric ventricles (see dashed line and arrows in A). Associated to ventricular enlargements were obvious signs of asymmetric gliosis (B) as demonstrated by extensive GFAP staining in proximity to the ependymal layer. Focal leakage of the blood-brain barrier was demonstrated in the periventricular regions by FITC albumin microangiography (arrows in A). The dashed lines indicate the pial surface (pm, pia mater); cx, cortex.
lower than those seen prior to pronounced EEG changes or after exposure to lithium alone (no SE), suggesting that increased IL-1β levels were not due to seizure activity. However, after SE, Il-1β serum level remained elevated compared to control. EEG changes observed after LiCl treatment occurred at the time corresponding to the ongoing peripheral pro-inflammatory process and BBB damage (see Figs. 2–6).

Since elevated IL-1β serum levels (prior SE) is a hallmark of the pilocarpine and lithium–pilocarpine models and since IL-1β is a recognized mediator of inflammatory BBB failure (Cucullo et al., 2008; Shaftel et al., 2007), we tested the efficacy of preventive anti-IL-1β treatment on seizure generation in the convulsive model of SE induced by 320 mg/kg of pilocarpine. The notion that anti-inflammatory therapy may be useful in the treatment of SE is also supported by clinical data demonstrating that SE may be terminated or prevented by exposure to steroids or other anti-inflammatory drugs of similar potency (see below and Vezzani and Granata, 2005). To achieve blockade of downstream effects triggered by IL-1β, we measured the prevalence of SE in animals pre-treated with the recombinant rat IL-1 receptor antagonist (IL-1ra; Eisenberg et al., 1991) 3.5–35 μg/kg) or saline. The data shown in Fig. 7 demonstrate a significant reduction in SE onset by the anti-inflammatory treatment delivered intravenously 2 h before pilocarpine. In the remaining animals, where seizure nevertheless occurred, these were reduced in intensity as assessed behaviorally (Fig. 7C). At low dosage (3.5 μg/kg), IL-1ra failed to protect against SE.

Fig. 6. Lithium induces rapid activation of circulating white blood cells. White blood cells were separated in CD3 positive and negative cells to quantify activation of T-lymphocytes (CD3+) or monocyte/macrophages (CD3−). (A, B) Within the CD3 negative population (granulocytes/monocytes) the expression of the surface marker CD11b was increased compared to control. CD11b interacts with intercellular adhesion molecule 1 expressed endogenously on endothelial cells to stabilize the adhesion of leukocytes to endothelium, facilitating the recruitment of leukocytes from the circulation into tissue. (C) Rapid and significant decrease in the CD4:CD8 ratio after lithium treatment reflects increased cytotoxic T-cell activity. CD3 is a three-subunit complex expressed by mature T cells. Mature T cells express either CD4 or CD8. CD4, a member of the Ig supergene family, is a single-chain transmembrane glycoprotein. CD4 and CD8 act as co-receptors during T cell activation. CD4 is expressed on the surface of helper cells, while CD8 is predominantly expressed on the surface of cytotoxic T cells, but can also be found on natural killer cells. (D) Rapid increase in IL-1β following lithium administration. Note that serum IL-1β levels after lithium were comparable to those seen after a convulsive dose of pilocarpine. Following SE, the levels of IL-1β were reduced, implying that the reported surge in IL-1β after SE (Marchi et al., 2007b) are the tail of pre-seizure increase rather than a consequence of SE itself (Vezzani and Granata, 2005; Cucullo et al., 2008).
The results so far presented pointed to blood-brain barrier disruption as a mechanism of pilocarpine-induced SE. If this hypothesis were correct, one expects that preventing leakage across the BBB may indeed protect against epileptic seizures. In other words, it was reasonable to expect that in animals where SE is prevented by IL-ra, BBB leakage would be reduced. This was directly tested by quantifying the extent of BBB extravasation of FITC albumin, as shown in Figs. 7D, E. Note that the pre-treatment with IL-ra caused a dramatic and ubiquitous reduction of BBB leakage; in addition, the extent of this effect on the BBB correlated with the seizure-preventing actions of IL-ra (D).

**Discussion**

Common risk factors for epilepsy are CNS infections, CNS malignancies and head injury; all these are accompanied by variable levels of inflammation and cerebrovascular dysfunction, a possible etiological trigger for seizures (Vezzani and Granata, 2005; Marchi et al., 2007a). In agreement with a role for inflammation in determining propensity to seizures is anecdotal evidence that anti-inflammatory drugs are useful in treating refractory seizures (see Vezzani and Granata, 2005). Our results have extended these findings and also provide a mechanistic hypothesis to explain how these non-traditional antiepileptic drugs may exert their action. We have shown that anti-inflammatory therapy (by inhibition of IL-1β signaling) is an effective strategy to prevent SE induced by pilocarpine.

We have shown that in cholinergic animal models of SE (pilocarpine, lithium–pilocarpine, and pilocarpine–lithium), white blood cell activation and an early surge in serum IL-1β levels precede seizure onset. This was also true for seizure-facilitating, yet non-convulsive treatments such as lithium exposure. Thus, as in the traditional model of TLE induced by pilocarpine exposure (Turski et al., 1989), intravascular pro-inflammatory changes are prodromic events to acute seizures. Human and animal studies demonstrated that breaching the BBB is sufficient to trigger seizures (Marchi et al., 2007a). The seizure-promoting manipulations described here similarly increased BBB permeability to serum protein. Thus, the common final pathway linking systemic inflammation to seizures appears to be loss of cerebrovascular control of brain homeostasis. Blockade of this cascade upstream from BBB leakage by IL-1ra prevented the development of pilocarpine-induced seizures.
We are well aware that the predictive value of therapeutic antiepileptic treatments extrapolated from animal models is limited. For example, we and others have shown that multiple drug resistance to antiepileptic drugs is a complex phenomenon that is difficult to model experimentally (Abbott et al., 2001; Dombrowski et al., 2001; Oby and Janigro, 2006). In addition, for most of the animal experiments described here we used a specific cholinergic trigger to tilt the equilibrium of brain activity towards status epilepticus. The question thus remains as to the therapeutic potential of an anti-inflammatory strategy discussed above in human subjects affected by multiple drug resistant seizures. In other words, does anti-inflammatory therapy benefit patients affected by SE? A preliminary human study shown in the Supplemental information demonstrates a dramatic efficacy of steroidal immunomodulators in the treatment of SE in children with refractory SE (For pharmacological regimens and information please see Supplemental Information and Figure therein). These results show a similar efficacy (>80%) in pediatric epilepsy regardless of the corticosteroid treatment used (prednisolone, ACTH, hydrocortisone or dexamethasone).

The use of steroids to treat pediatric seizures where an inflammatory etiology has not been reported or suspected has never been systematically studied. This is due in part to the fact that the triggers of idiopathic paroxysmal events are not known. Only recently systemic inflammation emerged as a possible target for a broad spectrum of seizure disorders. However, the mechanisms by which steroids or other immunomodulators reduce seizures are not fully understood. A possible direct action on CNS neurons has long been suspected, and recent findings were interpreted as in identical to the one previously observed after β

We demonstrated that lithium triggered an increase in CD11b expression which is a mechanism of increased leukocyte-endothelial adhesion (Wertheimer et al., 1992). While pilocarpine specifically acts on muscarinic receptors, lithium was demonstrated to interact with a number of signal transduction pathways. At concentration similar to those used in our experiments, lithium inhibits glycogen synthase kinase-3β (GSK-3β). Inhibition of GSK-3β determines a downstream increase in the transcription of vascular endothelial growth factor (VEGF), the chemokine receptor CXCR4 and the chemokine CXCL12. CXCR4 immunoreactivity has been reported in vascular endothelial cells and is involved in cellular trans-migration and, together with VEGF, in the modification of vascular permeability (Kaga et al., 2006; Skurk et al., 2005). All these findings suggest that regardless of the initiator of chemokine surge, a predictable downstream effect involving the BBB will occur. Thus, while the findings presented herein involve IL-1β as a serological predictor of seizures, the blood-brain barrier failure could be the ultimate etiological event.

When the results shown in Figs. 3 and 4 were merged this revealed that lithium induced BBB damage at time of increased theta EEG activity (Ulrich et al., 1987; Suslow et al., 2004). These alterations of BBB permeability were observed prior to onset of status epilepticus, suggesting that pronounced systemic inflammation leads to BBB impairment. Recent evidences also support a role for BBB failure in the pathogenesis or exacerbation of seizures, strengthening the hypothesis that BBB damage induced by LiCl is responsible for increased theta activity and lowered threshold for pilocarpine-triggered SE.

The possibility exists that a brain inflammatory response was also present at the time of SE and that the effects of IL-1β were mediated in part by inhibition of CNS cytokine signaling. Even though the involvement of IL-1β/IL-1ra in epilepsy has been proposed (Vezzani and Granata, 2005), our experimental paradigm differs significantly from those used for example by Vezzani and colleagues (Vezzani et al., 2000). In our work, chemical seizure triggers were injected intraperitoneally, therefore being first presented to circulating white blood cells and the BBB. In contrast, previous studies were performed administering the pro-epileptogenic trigger directly into the CNS. We have also excluded that the surge of serum IL-1β level could result from CNS activation for the following reasons: 1) the seizure trigger was administered systemically; 2) the cytokine surge occurred concomitantly to activation of circulating white blood cells; 3) this activation was fast and occurred well before onset of SE in the pilocarpine model and within 3 h from the injection of LiCl; at these time points no seizures activity was detected by EEG; 4) we observed no evidence of limbic gliosis, since periventricular glial activation was not present at the time of SE and that the effects of IL-1ra were mediated in part by decreased systemic permeability of the blood-brain barrier induced by LPS (Sayyah et al., 2003; Veldhuis et al., 2003) or seizure-promoting agents such as lithium (our results). We demonstrated that the pro-epileptogenic effects of a cholinergic agonist (pilocarpine) or lithium are surprisingly similar to those mediated by systemic infection. Infections (CNS or peripheral) are well-recognized etiological triggers for seizures, suggesting that the pilocarpine model of SE is much more clinically significant now that a systemic inflammatory cascade induced by cholinergic agents has been unveiled. This is supported by the fact that systemic inflammation is a common trigger for acute seizures in pediatric and adult patients worldwide (Vezzani and Granata, 2005).

We demonstrated that lithium triggered an increase in CD11b expression which is a mechanism of increased leukocyte-endothelial adhesion (Wertheimer et al., 1992). While pilocarpine specifically acts on muscarinic receptors, lithium was demonstrated to interact with a number of signal transduction pathways. At concentration similar to those used in our experiments, lithium inhibits glycogen synthase kinase-3β (GSK-3β). Inhibition of GSK-3β determines a downstream increase in the transcription of vascular endothelial growth factor (VEGF), the chemokine receptor CXCR4 and the chemokine CXCL12. CXCR4 immunoreactivity has been reported in vascular endothelial cells and is involved in cellular trans-migration and, together with VEGF, in the modification of vascular permeability (Kaga et al., 2006; Skurk et al., 2005). All these findings suggest that regardless of the initiator of chemokine surge, a predictable downstream effect involving the BBB will occur. Thus, while the findings presented herein involve IL-1β as a serological predictor of seizures, the blood-brain barrier failure could be the ultimate etiological event.

When the results shown in Figs. 3 and 4 were merged this revealed that lithium induced BBB damage at time of increased theta EEG activity (Ulrich et al., 1987; Suslow et al., 2004). These alterations of BBB permeability were observed prior to onset of status epilepticus, suggesting that pronounced systemic inflammation leads to BBB impairment. Recent evidences also support a role for BBB failure in the pathogenesis or exacerbation of seizures, strengthening the hypothesis that BBB damage induced by LiCl is responsible for increased theta activity and lowered threshold for pilocarpine-triggered SE.

The possibility exists that a brain inflammatory response was also present at the time of SE and that the effects of IL-1β were mediated in part by inhibition of CNS cytokine signaling. Even though the involvement of IL-1β/IL-1ra in epilepsy has been proposed (Vezzani and Granata, 2005), our experimental paradigm differs significantly from those used for example by Vezzani and colleagues (Vezzani et al., 2000). In our work, chemical seizure triggers were injected intraperitoneally, therefore being first presented to circulating white blood cells and the BBB. In contrast, previous studies were performed administering the pro-epileptogenic trigger directly into the CNS. We have also excluded that the surge of serum IL-1β level could result from CNS activation for the following reasons: 1) the seizure trigger was administered systemically; 2) the cytokine surge occurred concomitantly to activation of circulating white blood cells; 3) this activation was fast and occurred well before onset of SE in the pilocarpine model and within 3 h from the injection of LiCl; at these time points no seizures activity was detected by EEG; 4) we observed no evidence of limbic gliosis, since periventricular glial activation was most prevalent; 4) finally, the increase in serum IL-1β (up to ~300 pg/ ml) is well above the CNS production observed when kainic acid is injected directly in the hippocampus (24 h after seizures IL-1β ~20 pg/ mg in the injected hippocampus only, Vezzani et al., 1999). In our experimental paradigm, we sacrificed the animal before or at the onset of SE. This excludes a possible contribution of seizure activity to the production of IL-1β. Moreover, we have recently demonstrated that BBB opening induced by osmotic shock is sufficient to trigger immediate focal motor seizures in humans, pig and rats, therefore excluding CNS inflammation as a requirement for the development of seizures (Marchi et al., 2007a). Nevertheless, CNS inflammation is...
observed in chronic epileptic brain tissue and may be involved in the process of establishment and sustaining of the epileptic pathology.

The role for BBB leakage in epileptic seizures has been long suspected but only recently has clinical and experimental evidence converged to the demonstration of a clear etiologic role. In human subjects, acute BBB disruption (Marchi et al., 2007a; Elkassabany et al., 2008) (Korn et al., 2005) induces seizures. In animal models, chronic (Ivens et al., 2007) or acute (Fieschi et al., 1980) BBB disruption also lead to seizures, sometimes progressing into full blown epilepsy (Van Vliet et al., 2007). The results presented here again support the notion that blood-brain barrier disruption is a powerful seizure promoting event. However, we here extended this concept by directly correlating the extent of BBB to the severity of seizures (Figs. 7D, E). As the data clearly show, seizures occurred only when the BBB was significantly breached, and, conversely, IL-ra was effective when BBBD was prevented. An apparent threshold effect was also noted, since the prodromic seizure-like events (stages 2-4) were not associated with quantifiable blood-brain barrier disruption. This is in agreement with the reported cholinergic effects of pilocarpine consisting of cholinergic gamma oscillations independent from blood-brain barrier disruption (Uva et al., 2006). The finding that extent of BBB “opening” correlates with the probability of seizure occurrence was also reported in human subjects; while animal and patient data are clearly mutually supportive, it has to be underscored that the methods used to detect BBB leakage were different (radiologic/serologic vs. albumin extra-vasation). The fact that these different techniques yielded essentially to the same results supports the power of S100β and FITC-albumin as experimental markers of BBB function.

What clearly remains to be elucidated are why BBB disruption is noxious and under which clinically relevant conditions these disruptions occur. In addition to iatrogenic blood-brain barrier disruption used to treat brain tumors (Kroll and Neuwelt, 1998), several pathologies lead to BBB dysfunction. These include traumatic brain injury, stroke, and various infectious diseases (Grant and Janigro, 2004). All these human pathologies dramatically decrease seizure threshold (Hesorffer, 2008). Treatment with anti-inflammatory steroids has a profound impact on cerebrovascular integrity (Ostergaard et al., 1999) by acting on immune cells and by a direct action on cerebrovascular endothelial cells (Cucullo et al., 2004). Interestingly, a recent clinical study reported that corticosteroids reduce seizure frequency during invasive monitoring (Araki et al., 2006), suggesting that modulation of paroxysmal activity by anti-inflammatory drugs is a realistic approach. The steroidal therapy used for the patients shown in Supplemental Information was beneficial if one bears in mind the devastating history of drug resistant SE. However, the use of anti-inflammatory steroids is not devoid of side effects, and prolonged steroid use is rarely possible. The novel approach in our animal study consisted of using a downstream target such as IL-1β. While this is an entirely new concept in antiepileptic drug therapy, we believe that the results presented here will prompt further studies aiming at cerebrovascular protection by modern anti-inflammatory drugs.

Another point of caution relates to the comparison of the patients’ data presented in Supplemental Information and the animal data presented in this manuscript. The immune system and its associated inflammatory reactions have been implicated, by numerous experimental and clinical findings, in the pathogenesis of several forms of epilepsy. The relationship between epilepsy, the immune system, and the inflammatory cascade is, however, exceedingly complex and possibly bidirectional. Thus, the use of steroids in pediatric epilepsy is not entirely novel (Kokate et al., 1996; Hrachovy and Frost, 2008). What is different in this study is the fact that the patients treated with steroids did not belong to the therapeutic categories that are usual recipients of steroidal therapy. For example, we intentionally excluded from this study patients with infantile spasms or other pathologies with a clear cut infectious or inflammatory diagnosis (Vezzani and Granata, 2005; Granata, 2003). In addition, and for the same reasons, patients with Rasmussen’s syndrome were similarly excluded (Granata, 2003). The novelty of this approach therefore rests with applying an established treatment to an entirely new target population of patients affected by epilepsy and multiple drug resistance. In addition, translation of the steroidal treatment in epileptics to the use of IL-1β in an animal model of limbic epilepsy may also be problematic. However, there are several points of convergence between these sets of findings. First and foremost, all steroids used in our human study target cytokine production and are excellent modulators of IL-1β in particular (e.g., Vezzani and Granata, 2005; Vezzani and Janigro, 2008). In addition, steroids have been shown to prevent pilocarpine epileptogenesis in rats (Kokate et al., 1996). Thus, these two models of antiinflammatory treatment of epilepsy are closely related and potentially significant.

Taken together, the animal studies presented here suggest that SE may be triggered by systemic inflammatory events and BBB damage. This was supported by clinical studies showing that immunomodulators are a useful tool against SE. While the animal studies presented here were limited to the pilocarpine models, the general relevance to seizure disorders will require further experimental evidence.

Acknowledgments

This work was supported by NIH-R01 NS43284 NIH-R01 NS38195 to Damir Janigro. We would like to thank Drs. P.A. Schwartzkroin and R. M. Ransohoff for reviewing the manuscript and giving help and encouragement.

Appendix A. Supplementary data


References


Bawden, H.N., Racine, R.J., 1979. Effects of bilateral kindling or bilateral sub-threshold stimulation of the amygdala or septum on muricide, ranacide, intrapsychic aggression and passive avoidance in the rat. Physiol. Behav. 22, 115–123.


Hesdorffer, D.C., 2008. Treatment with anti-in-