Original Paper

Inhibiting HIF-1 α Decreases Expression of TNF- α and Caspase-3 in Specific Brain Regions Exposed Kainic Acid-Induced Status Epilepticus

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Key Words

HIF-1 α • Cytokines • Epilepsy • Neural dysfunctions • Caspase-3 • Kainic acid • Parietal cortex • Hippocampus

Abstract

Background/Aims: A recent study demonstrates that pro-inflammatory cytokines (PICs, i.e., IL-1 β , IL-6 and TNF- α) in specific brain regions of rats play a role in regulating kainic acid (KA)-induced status epilepticus (SE) via a GABAergic mechanism. The purposes of this report were to examine contributions of hypoxia inducible factor subtype 1 α (HIF-1 α) to expression of PICs in these specific brain regions in epileptic rats. Particularly, we investigated the parietal cortex, hippocampus and amygdala. In addition, we further examined expression of Caspase-3 indicating cell apoptosis in those brain regions of epileptic rats after infusing 2-methoxyestradiol (2-MET, inhibitor of HIF-1 α) and etanercept (TNF- α receptor antagonist).

Methods: ELISA was used to determine the levels of HIF-1 α and PICs and western blot analysis was used to examine Caspase-3 expression. **Results:** Our data show that HIF-1 α was significantly increased in the parietal cortex, hippocampus and amygdala 1, 3 and 7 days after induction of SE ($P < 0.05$ vs. control rats). Our results also show that inhibiting HIF-1 α by central infusion of 2-MET significantly decreased the amplified TNF- α expression in these brain regions evoked by SE ($P < 0.05$ vs. vehicle control), but did not modify IL-1 β and IL-6. Our results demonstrate that 2-MET and etanercept attenuated an increase in Caspase-3 evoked by SE. **Conclusion:** Overall, we suggest that HIF-1 α activated by SE is likely to contribute to epileptic activity via a TNF- α pathway, which has pharmacological implications to target specific HIF-1 α and TNF- α pathways for neuronal dysfunction and vulnerability related to epilepsy.

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Introduction

It is well documented that abnormal patterns of highly synchronized recurrent neuronal discharges are observed in one or more brain areas as epilepsy occurs, which frequently recruit anatomically related structures [1]. In general, seizures are relatively brief, as in the complex partial seizures of temporal lobe epilepsy; however, in the case of status epilepticus (SE), seizures may persist for minutes or hours, and this may culminate in neuronal damage within several limbic and cortical areas [2]. Nevertheless, neural substrates responsible for epileptic activities remain to be determined.

Hypoxia inducible factor-1 (HIF-1) as an important endogenous signaling protein contributes to physiologic changes of homeostasis under conditions of oxygen deprivation [3, 4]. Accumulated subunit HIF-1 α in the cellular nucleus and cytoplasm modulates the expression of several target genes which are involved in neuroprotection, erythropoiesis, and apoptosis modulation [5, 6]. Additionally, inadequate oxygen supply is likely to appear in the brain regions during SE, which is associated with seizure-evoked behavioral activities [7, 8]. Recent studies have demonstrated that hypoxia markers including HIF-1 α are expressed in pathological brain tissue from patients affected by temporal lobe epilepsy as well as in animal models of this type of epilepsy [9-11], indicating that HIF-1 α is involved in hippocampal apoptosis after induction of SE.

In light of the key role of HIF-1 α in response to lack of oxygen, in this study we examined responsiveness of HIF-1 α in the representative brain regions related to epileptic activity, namely the parietal cortex, hippocampus and amygdala after induction of SE.

It has been reported that IL-1 β , IL-6 and TNF- α in plasma/cerebrospinal fluid and cells immunoreactive to those proinflammatory cytokines (PICs) in some tissues are increased in patients with temporal lobe epilepsy [12, 13], this is consistent with a role for PICs in engagement of the pathophysiology of epilepsy and/or brain damage following seizure. Moreover, in animals with SE induced by hippocampal kainic acid (KA) injections or by electrical stimulation increases mRNA expression of hippocampal IL-1 β , IL-6 and TNF- α [14, 15]. Because SE induces relatively widespread brain damage, evaluation of the functional protein levels of PICs production in the local brain regions during and/or following SE is more important. A recent study has demonstrated that protein levels of IL-1 β , IL-6 and TNF- α are significantly increased in the parietal cortex, hippocampus and amygdala after induction of KA-evoked SE [16].

It should be noted that there are a number of animal models widely used to study the mechanisms of complex partial epilepsy and development of new antiepileptic drugs [17]. For example, systemically applied drugs (e.g., pilocarpine preceded by lithium treatment) and neurotoxins (e.g., kainic acid) as well as kindling have typically been used to study SE.

In this report, a rat model of cerebral KA injection-induced SE [18-20] was employed and we also determined the effects of inhibiting HIF-1 α on the levels of PICs in the parietal cortex, hippocampus and amygdala. Moreover, we examined the effects of inhibiting of HIF-1 α and TNF- α receptor on protein expression of an indicator of cell apoptosis Caspase-3 in those specific brain regions of epileptic rats. We hypothesized that KA-induced epilepsy activates HIF-1 α in the parietal cortex, hippocampus and amygdala thereby leading to upregulation of PICs pathways in these specific brain regions and consequent neuronal damages.

Materials and Methods

All animal protocols of this study were conducted in conformity with the institutional guidelines in accordance with the International Association for the Study of Pain and NIH regulations. Male Wistar rats weighing 200-250 g were used in our experiments and all animals were maintained under 12/12 h light/dark-cycle with free access to food and water in a temperature- and humidity- controlled room.

General Surgery and Administration of Drugs

Rats were anesthetized with chloral hydrate (40 mg/kg body weight, i.p.), then immobilized in a stereotaxic apparatus (David Kopf, USA). After making a midline incision, the skull was exposed and one

burr hole was drilled. Following this, animals were cannulated with an L-shaped stainless steel cannula aimed at the lateral ventricle according to the coordinates: 3.7 mm posterior to the bregma, 4.1 mm lateral to the midline, and 3.5 mm under the dura. The guide cannula was fixed to the skull using dental zinc cement and jewelers' screw. After animals recovered from anesthesia, in order to induce status epilepticus they were injected with 1.0 μ l of KA [0.5 μ g in 1.0 μ l of artificial cerebrospinal fluid (aCSF)] in the lateral ventricle as described previously [20]. The rats were injected with the same volume of aCSF as controls.

Then, animal behaviors were observed. Consistent with the prior report [20], rats developed status epileptic and convulsive seizures within ~30 min after KA injection, which lasted at least for 1–2 hrs. Also, during seizure behavior animals showed rolling toward one side, rotating, and stiffing of limbs and tail but no movement during the interphase of spasms. In the control group, rats with aCSF injection did not exhibit any of these abnormal behaviors. It should be noted that all the rats that developed those abnormal seizure behaviors after KA injection were included in this study.

In a subset of groups, the cannula was connected to an osmotic minipump (Alzet pump brain infusion kit, DURECT Inc., Cupertino, CA) with polycarbonate tubing. The pumps were placed subcutaneously between the scapulae, and loaded with vehicle (aCSF) as control or 100 μ g of 2-methoxyestradiol (2-MET, a HIF-1 α inhibitor, Sigma-Aldrich) and 10 μ M of etanercept (ETAN, TNF- α receptor antagonist, Tocris Co.), respectively. 2-MET and ETAN were delivered at 0.25 μ l per hour (Alzet Model 1003D/3 day-delivery DURECT Inc., Cupertino, CA). This intervention allowed animals to receive continuous intracerebroventricular (ICV) infusion via the osmotic minipumps before brain tissues were removed.

Accordingly, rats were divided into sham control group with aCSF; epileptic group induced by KA injection followed by ICV infusion of aCSF; and epileptic group induced by KA injection followed by ICV infusion of respective 2-MET and ETAN. After completion of those treatments, all the animals were anesthetized and sacrificed 1, 3 and 7 days after KA-injection for the experiment to examine time courses of HIF-1 α response; and 3 days after KA for other experiments. The brains were removed to confirm the placement of the cannula, and then the brain regions were dissected under an anatomical microscope and tissues were taken for the process.

ELISA Measurements

The levels of HIF-1 α were determined using an ELISA assay kit (Abcam Co.) according to the provided description and modification. Briefly, polystyrene 96-well microtiter immunoplates were coated with affinity-purified polyclonal rabbit anti-HIF-1 α antibody. Parallel wells were coated with purified rabbit IgG for evaluation of nonspecific signal. After overnight incubation at room temperature and 2 hours of incubation with the coating buffer containing 50 mM carbonate buffer (pH 9.5) in 2% BSA, plate were washed with 50 mM Tris-HCl. After extensive washing, the diluted samples and the HIF-1 α standard solutions were distributed in each plate and left at room temperature overnight. The plates were then washed and incubated with anti-HIF-1 α galactosidase per well. Then, the plates were washed and incubated with substrate solution. After an incubation of 2 hours at 37°C, the optical density was measured using an ELISA reader. Likewise, this method was also employed to examine the levels of IL-1 β , IL-6 and TNF- α according to the provided description and modification (Promega Corp. Madison, WI).

Western Blot Analysis

The brain tissues were removed, homogenized, centrifuged and incubated with streptavidin beads. The beads were washed and precipitated by centrifugation and sample buffer was added to the collected beads. Beads were pelleted again by centrifugation to obtain supernatant. The supernatant was then diluted to the same volume and applied to SDS-PAGE. Membranes were incubated with the rabbit anti-Caspase-3 primary antibody (1:500, Neuromics) and goat anti-rabbit secondary antibody (1:500). Immunoreactive proteins were detected by enhanced chemiluminescence. The membrane was also processed to detect β -actin for equal loading. The densities of protein bands were analyzed using the Scion image software.

Statistical Analysis

All data were analyzed using a two-way repeated-measures analysis of variance. Values were presented as means $\pm$ standard error of mean (SEM). For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS for Windows version 13.0 (SPSS, USA).

Results

Levels of HIF-1 α

Figure 1 shows that the levels of HIF-1 α were significantly increased in the parietal cortex, hippocampus and amygdala of KA-rats ($P < 0.05$ vs. control rats, $n = 10-12$ in each group) as compared with sham control group ($n = 10-12$ in each group). The increases were seen 1, 3 and 7 days after induction of SE and the highest levels were observed 3 days after SE.

Effects of 2-MET on PICs

Figure 2 demonstrates that IL-1 β , IL-6 and TNF- α were significantly elevated in the parietal cortex, hippocampus and amygdala 3 days after KA injection ($n = 10$, $P < 0.05$ vs. control rats) as compared with control rats ($n = 10$). Inhibiting HIF-1 α by infusing 2-MET significantly decreased expression of TNF- α in the parietal cortex, hippocampus and

Fig. 1. The levels of HIF-1 α in the parietal cortex (PCX), hippocampus (HIP) and amygdala (AMG) 0, 1, 3 and 7 days after induction of SE. HIF-1 α was significantly increased in SE-rats 1, 3 and 7 days after cerebral KA injection as compared with control animals. Data are expressed as mean $\pm$ SEM. * $P < 0.05$, indicated rats with KA-induced SE ($n = 10-12$ in each group) vs. control rats ($n = 10-12$ in each group).

Fig. 2. Expression of IL-1 β , IL-6 and TNF- α in the parietal cortex (PCX), hippocampus (HIP) and amygdala (AMG). The cytokines were significantly increased in SE-rats 3 days after cerebral KA injection as compared with control animals. Inhibiting HIF-1 α by infusing 2-MET into the lateral ventricle (100 μ g) significantly decreased the levels of TNF- α in the parietal cortex, hippocampus and amygdala, but not the levels of IL-1 β and IL-6 in those brain regions. Data are expressed as mean $\pm$ SEM. * $P < 0.05$, indicated rats with KA-induced SE ($n = 10$) vs. control rats ($n = 10$). # $P < 0.05$, indicated rats with KA-SE with 2-MET injection vs. rats with KA-SE with aCSF as control ($n = 12$ in each group).

Fig. 3. Expression of Caspase-3 in the parietal cortex (PCX), hippocampus (HIP) and amygdala (AMG). Caspase-3 was significantly increased in SE-rats ($n = 12$) 3 days after cerebral KA injection as compared with control animals ($n = 10$). Blocking HIF-1 α (panel A) and TNF- α receptors (panel B) by respective lateral ventricular infusion of 2-MET ($n = 12$) and etanercept (ETAN, $n = 10$) decreased the effects of KA-induced SE on Caspase-3. Data are expressed as mean $\pm$ SEM. * $P < 0.05$ vs. control rats and KA injection with the prior 2-MET and ETAN. Top panels show typical bands that are representative of expression of Caspase-3 in the PCX region of three groups of rats. Bottom panels are averaged data.

amygdala ($P < 0.05$, SE rats with 2-MET vs. SE rats with aCSF, $n = 12$ in each group). Infusing 2-MET tended to attenuate expression of IL-1 β and IL-6 in those brain regions, but there were no significant differences observed ($P > 0.05$, SE rats with 2-MET vs. SE rats with aCSF).

Protein Expression of Caspase-3

Figure 3 illustrates that the protein expression of Caspase-3 in the parietal cortex, hippocampus and amygdala was significantly greater in KA-rats 3 days after KA injection ($n = 12$, $P < 0.05$ vs. control animals) than those in control rats ($n = 10$). Also, attenuating HIF-1 α ($n = 12$) and TNF- α receptors ($n = 10$) by individual infusion of 2-MET and ETAN significantly decreased the effects induced by KA injection on the expression of Caspase-3 in the parietal cortex, hippocampus and amygdala. Insignificant differences were observed in Caspase-3 expression between control animals and animals infused with 2-MET and ETAN, respectively.

Discussion

A rat model of cerebral KA injection-induced SE was employed to further investigate the mechanisms responsible for epileptic disease and antiepileptic drugs in the previous studies [18-20]. In the current study, using the same rat model our data showed that HIF-1 α was significantly increased in the specific brain regions such as the parietal cortex, hippocampus and amygdala 1-7 days after induction of SE by cerebral KA injection (Fig. 1). It has been reported that PICs are involved in later phase alterations of neuronal damage

and electroencephalography related to epileptic activity and significant increases in IL-1 β , IL-6 and TNF- α in hippocampus were observed 3 days following KA injection [20]. Our data further demonstrated that KA-induced epilepsy increased the levels of IL-1 β , IL-6 and TNF- α in those specific brain regions (Fig. 2). The time course for KA injection to increase HIF-1 α and PICs is similar. Moreover, our data showed that an inhibition of HIF-1 α significantly attenuated the amplified TNF- α in those brain regions; however, the effects of inhibiting HIF-1 α on IL-1 β and IL-6 were not significant (Fig. 2). In addition, Caspase-3 as an indicator of neuronal apoptosis was examined in this study and we found that KA increased Caspase-3 in the parietal cortex, hippocampus and amygdala 3 days after induction of SE (Fig. 3). Inhibiting cerebral HIF-1 α and TNF- α decreased the amplified expression of Caspase-3 evoked KA injection. Overall, we suggest that enhancement of HIF-1 α activity in those specific brain regions contribute to the neuronal damage via TNF- α mechanisms during epileptogenesis.

Elevated levels of PICs in the brain are associated with increased seizure vulnerability and seizure-related pathological changes such as neuronal death [13, 21, 22], suggesting that inflammation has been implicated in epileptogenesis. In a rat model of KA-induced epilepsy, neural injuries in the brain occur and this is accompanied by upregulation of PICs including IL-1 β , IL-6 and TNF- α in glial cells [20, 23]. Furthermore, neuronal degeneration is observed likely due to increases of PICs [24, 25]. For example, IL-1 β can increase seizure susceptibility in rat brains [26]. Intracerebral injection of a high dosage of IL-1 β results in limbic seizures in wild type mice, but not in transgenic mice with deficient IL-1 β receptors [27]. In addition, antiepileptic activity is observed after using thalidomide to decrease the levels of TNF- α [28]. With respect to the role played by central IL-6 in regulating epileptic activity, prior studies demonstrate that IL-6 can stimulate the synthesis of corticotrophin and glucocorticoids, and initiate an anti-inflammatory feedback loop, suggesting a neuroprotective role played by IL-6 [29, 30]. Consistent with the findings of prior studies [31-33], we have observed increases in the levels of IL-1 β , IL-6 and TNF- α in the parietal cortex, hippocampus and amygdala 3 days after KA-induced SE.

Prior studies in humans and animal models have demonstrated that temporal lobe epilepsy is generally associated with an initial brain insult such as neuronal loss in the hippocampus during epileptogenesis [34-36]. It is indicated that apoptosis is engaged in the pathological process of seizure-induced neuronal loss in epilepsy, and a greater number of neurons that are stained with terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) are found in hippocampus [37]. Repeated administration of ketamine was also reported to induce hippocampal neurodegeneration and long-term cognitive impairment via the ROS/HIF-1 α pathway in developing rats [38]. Also, sequential activation of Caspase-3 plays an important role in the execution-phase of cell apoptosis linked to neuronal death in epileptic model [37]. Nevertheless, as an important factor in mediating neuronal apoptosis the role played by HIF-1 α in engagement of epileptic activities is lacking. Data of the current study suggest that HIF-1 α in the parietal cortex, hippocampus and amygdala is likely to contribute to the pathophysiological process in KA injection-induced SE. Because our results show that inhibiting HIF-1 α and TNF- α significantly decreased expression of Caspase-3 in those brain regions of SE rats, we further suggest that there are likely pathways of HIF-1 α and TNF- α to be involved in the process of epilepsy.

In the current study, the significant effects of inhibiting HIF-1 α on the levels of IL-1 β and IL-6 in the parietal cortex, hippocampus and amygdala were not observed. The underlying mechanisms responsible for this discrepancy are unclear. We assume that HIF-1 α activated by SE stimulates different signaling pathways in the cellular nucleus and cytoplasm that regulate expression of PICs due to different HIF-1 α -targeted genes [5, 6].

It should be addressed that in the current study we did not investigate the cell type(s) expressing Caspase 3, in general neurons should be the prevalent population. However, we cannot exclude the possibility that astrocytes could be also involved because a prior study demonstrated disappearance of astrocyte in the kainate model [39].

Conclusions

We have provided evidence that KA-induced epileptic activities amplify the levels of HIF-1 α as well as IL-1 β , IL-6 and TNF- α in the parietal cortex, hippocampus and amygdala. In the process of KA-induced epilepsy, HIF-1 α leads to upregulation of TNF- α and this is involved in consequent neuronal damages in those brain regions. The results may offer promising clues for the development of new therapeutic strategies for managing intractable symptoms observed in patients with epilepsy.

Disclosure Statement

None.

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