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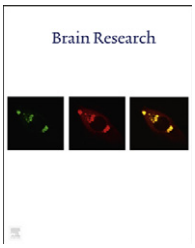

Research Report

Post-treatment of an NADPH oxidase inhibitor prevents seizure-induced neuronal death

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ABSTRACT

The present study sought to evaluate the neuroprotective effects of apocynin, an NADPH oxidase assembly inhibitor, on seizure-induced neuronal death. Apocynin, also known as acetovanillone, is a natural organic compound isolated from the root of Canadian hemp (*Apocynum cannabium*). It has been extensively studied to determine its disease-fighting capabilities and application in several brain insults, such as traumatic brain injury and stroke. Here we tested the hypothesis that post-treatment of apocynin may prevent seizure-induced neuronal death by suppression of NADPH oxidase-mediated superoxide production. Temporal lobe epilepsy (TLE) was induced by intraperitoneal injection of pilocarpine (25 mg/kg) in male rats. Apocynin (30 mg/kg, i.p.) was injected into the intraperitoneal space two hours after seizure onset. A second injection was performed 24 h after seizure. To test whether apocynin inhibits NADPH oxidase activation-induced reactive oxygen species (ROS) production, dihydroethidium (dHEt, 5 mg/kg, i.p.) was injected before onset of seizure and ROS production was detected five hours after seizure onset. Neuronal oxidative injury (4HNE), neuronal death (Fluoro Jade-B), blood brain barrier (BBB) disruption (IgG leak), neurotrophil infiltration (MPO) and microglia activation (CD11b) in the hippocampus was evaluated at three days after status epilepticus (SE). Pilocarpine-induced seizure increased p47 immunofluorescence in the plasma membrane of hippocampal neurons at 12 h post-insult and apocynin treatment prevented this increase.

Abbreviations: ANOVA, analysis of variance; ARRIVE, animal research reporting in vivo experiments; BBB, blood brain barrier; CA1, cornu ammonis area 1; CD11b, cluster of differentiation molecule 11b; DAB, diaminobenzidine; DMSO, dimethylsulphoxide; DPX, *p*-xylene-*bis*-pyridinium bromide permount; FJB, Fluoro-Jade B; 4HNE, 4-hydroxy-2-nonenal; i.p., intraperitoneal; NADPH, nicotinamide-adenine dinucleotide phosphate; MPO, myeloperoxidase; PBS, phosphate buffered saline; PFA, paraformaldehyde; RNS, reactive nitrogen species; ROS, reactive oxygen species; SE, status epilepticus

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The present study found that apocynin post-treatment decreased ROS production and lipid peroxidation after seizure and decreased the number of degenerating hippocampal neurons. Apocynin also reduced seizure-induced BBB disruption, neurotrophil infiltration and microglial activation. Taken together, the present results suggest that inhibition of NADPH oxidase by apocynin may have a high therapeutic potential to reduce seizure-induced neuronal dysfunction.

1. Introduction

Temporal lobe epilepsy (TLE) is one of the most devastating neurological disorders experienced by the young population and represents one of the leading causes of cognitive impairment (Chang et al., 2003; Holmes et al., 1998). The syndrome characterized by recurrent and unprovoked seizures is termed epilepsy. During epileptic seizure, the primary symptoms may include tonic-clonic movement or convulsions, accompanied by a brief or long-term loss of memory. Epilepsy encompasses a variety of disorders that reflects underlying brain dysfunction and can be induced by multiple causes, such as brain lesion after trauma and stroke. Severe epilepsy can lead to more serious secondary events such as oxidative stress, massive edema, and alteration of endogenous neurochemical processes that may be preventable if quickly hospitalized, but which can lead to complications if left untreated. Survival and recovery from severe epilepsy has been dramatically improved over the past few decades with modern clinical management practices, including antibiotic treatment and use of anticonvulsants drugs (Rogawski and Loscher, 2004). However, many survivors of severe epilepsy still display delayed neuronal death and permanent cognitive impairment. There is no currently available intervention for preventing this delayed neuronal death that develops even after the acute epilepsy is corrected, nor is the cause well-characterized.

Oxidative stress, caused by the imbalance between the generation and detoxification of reactive oxygen and nitrogen species (ROS/RNS), plays an important role in brain aging, neurodegenerative disease, and other related neurological conditions, such as ischemia. Reactive oxygen species (ROS) are involved in epilepsy-induced neuronal death (Frantseva et al., 2000; Freitas et al., 2005; Liu et al., 2010). Thus, free radical scavenging improves neuronal function after epilepsy (Ashrafi et al., 2007). The close relationship between epilepsy and oxidative stress has generated considerable interest in the development of antioxidant agents to combat the deleterious consequences of oxidative insult in epilepsy (de Freitas et al., 2010). Activated NADPH oxidases produce ROS, which are released into the intra- or extra-cellular space and contribute to progressive neuronal death (Bedard and Krause, 2007). NADPH oxidase is a multi-subunit enzyme complex present in numerous cell types. This enzyme is especially well-characterized in immune cells and leukocytes, where ROS production is utilized as a host-defense mechanism. However, it has been recently recognized that various protein subunits of NADPH oxidase are also expressed in neurons. In the brain, distinct distribution patterns have been described for some of the NADPH oxidase subunits, e.g., $gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ (Groemping and Rittinger, 2005). Due to the requirement of protein kinase activity for phosphorylation and translocation of NADPH oxidase subunits (e.g., $p47^{phox}$) prior to its activation, this enzyme is indirectly regulated by plasma membrane receptors and signaling pathways (Brennan et al., 2009). A recent study has implicated NADPH oxidase-derived ROS in modulating hippocampal function under both physiological and pathological conditions (Tejada-Simon et al., 2005). Additionally, our previous studies have demonstrated that NADPH oxidase-derived ROS production modulates neuronal death in hypoglycemia and ischemia (Suh et al., 2007, 2008a, 2008b). One previous study suggested an involvement of NADPH oxidase generated ROS in neuronal death after TLE (Pestana et al., 2010). In this study, treatment of rats with apocynin prior to induction of TLE decreased both ROS production and neurodegeneration. However, whether apocynin can prevent neuronal death if delivered after epilepsy has not yet been tested.

The present study used rodent models of pilocarpine-induced seizure to characterize the effectiveness of post-treatment with an NADPH oxidase inhibitor on neuronal death after TLE. The pilocarpine rat model has been regarded as one of the best models for studies of the relationship between epilepsy and the role of the hippocampus in TLE (Turski et al., 1983). We found that seizure-induced hippocampal neuronal oxidative injury, BBB disruption and microglial activation were prevented by inhibition of superoxide production through an NADPH oxidase-mediated pathway. Therefore, the present results suggest that post-treatment of apocynin may have a high therapeutic potential to reduce seizure-induced neuronal death.

2. Results

2.1. *Seizure-induced neuronal death is prevented by apocynin*

To test whether apocynin treatment shows neuroprotective effects after pilocarpine-induced seizure, rats were sacrificed 72 h after insult with or without apocynin injection. No degenerating neurons were detected by Fluoro-Jade B staining in the control group, with or without apocynin injection (DMSO, $n=3$; Apocynin, $n=3$). After pilocarpine injection, Fluoro-Jade B staining revealed degenerating neurons in CA1, CA3 and hilus area at three days after SE induction (Seizure+DMSO, $n=8$; Seizure+Apocynin, $n=10$). Post-treatment of apocynin after SE decreased the number of FJB (+) neurons in the CA1 by 33.4%, in the CA3 by 39.7% and in the hilus by 17.4% (Fig. 1).