

MOLECULAR INSIGHTS INTO THE ROLE OF INFLAMMATION AND OXIDATIVE STRESS IN EPILEPSY.

ABSTRACT

Epilepsy is a chronic neurological disorder characterized by innate acquired ability of neurons to generate recurrent seizures. Despite the use of current anti-epileptic drugs (AEDs) 30% of patients still remain refractory. One such lacuna in search for successful AEDs has been lack of understanding the processes leading to the cascade of epilepsy. Thus we tried to focus on role of inflammation and oxidative stress in epilepsy, it is well established that epileptic seizures result in increased expression of Toll like receptors, which increase the synthesis of cytokines, chemokines, complement system, transcription factors e.t.c. The epileptic seizures also damage blood brain barrier, increase influx of Ca^{2+} into cytoplasm, increase production of reactive oxygen species which brings imbalance between ROS/RNS all leading to excess inflammation, oxidative stress, neuronal degeneration and in fact all these changes also lead to epileptic seizures that feedback loop mechanism is involved.

Keywords: Inflammation; Oxidative stress; Toll like receptors; Blood brain barrier; Epilepsy

1.1 INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by recurrent seizures and is often accompanied by psychiatric disturbances (Jones et al., 2008). The excessive or synchronous neural activity in brain leads to epileptic seizures (Fisher et al., 2005). Epilepsy represents as a socioeconomic burden, usually patients suffering from epilepsy also suffer from social stigma. Epilepsy affects almost 70 million people of world (Ngugi et al., 2010). The incidence of epilepsy for low income and middle income countries is estimated as 81.7/100,000/year, while for high income countries it is estimated 45.0/100,000/year (Ngugi et al., 2011). Similarly the prevalence of epilepsy for low- and middle-income countries is about twice that of high-income countries (Ngugi et al., 2010). The median life time epilepsy prevalence for low income and middle income countries is 15.4 per 1,000 for rural and 10.3 per 1,000 for urban areas while for high income countries 5.8 per 1,000. similarly the median active epilepsy for low- and middle-income countries is 12.7 per 1,000 in rural and 5.9 per 1,000 in urban areas and for high income countries 4.9 per 1,000 (Ngugi et al., 2010). Insult from seizures or excitotoxic brain damage induce, activate and persist regional as well cellular patterns of inflammatory response to stimulate Toll like receptors (TLRs) which on activation stimulate

various genes encoding proinflammatory factors such as cytokines, chemokines, complement system, cyclooxygenase-2, nitric oxide via many transcriptional factors or nuclear factors such as nuclear factor κ B (NF κ B) resulting severe inflammation. The inflammatory responses induce the oxidative and nitrosative stress pathways, and subsequent mitochondrial metabolic processes generate highly reactive free radical molecules. High levels of intracellular calcium ions also induce reactive oxygen species (ROS) in a biosystem culminating in oxidative stress (Martinc et al., 2012). Hence in this review we have tried to focus on possible role of inflammation and oxidative stress in epilepsy and amelioration of which will offer neuroprotection to degenerating neurons, hence serve as novel approach for treatment of epileptic seizures.

2.1 INFLAMMATION IN EPILEPSY

Pathological condition of brain inflammation are distinguished from normal physiological conditions because parenchymal cells (microglia, astrocytes, and neurons), blood brain barrier (BBB) and choroid plexus mediate production of detectable quantity of many inflammatory mediators which are usually produced in our body by immune system either under pathological threats or in response to infection, and includes cytokine molecules as well as other inflammatory biomolecules. Rapidly and robust animal model for brain inflammation in rodents involves administering of systemic infection mimicking lipopolysaccharide (LPS); main structural components of not only external membrane of gram-negative bacteria but also molecular patterns of invading pathogenic microbes which are mainly recognized sites for specific receptors; Toll like receptors (TLRs) and cells of immune systems; followed by initiating characteristic and well distinguished inflammatory reactions so often explored for diagnostic purposes. These specific receptors; Toll like receptor (TLR) in presence of exogenous molecule get activated and stimulate various genes encoding proinflammatory factors such as cytokines, chemokines, complement system, cyclooxygenase-2, nitric oxide via many transcriptional factors or nuclear factors such as nuclear factor κ B (NF κ B) (Rivest, 2003). However apart from infectious agents, inflammatory responses in brain is also well predicted against various endogenous ligand and signals ; usually sourced from large spectrum of injuries, i.e., trauma , ischemia during seizures, or excitotoxic brain damage or damaged BBB or heat shock proteins of damaged extracellular matrix; so if these ligands enter the brain, depending upon injury level they may induce, activate and persist regional as well cellular patterns of inflammatory response to stimulate TLRs hence triggering of many immune responses at various time functions (Allan and Rothwell, 2001).

2.1.1 Different factors that mediate the influence of inflammation on epilepsy.

2.1.1.1 The role of blood brain barrier

CNS microvasculature are cellular basis of the blood brain barrier and made up of from non fenestrated endothelial cells with tight junctions made up of interendothelial cells. BBB maintenance and functioning is mainly regulated by the various type of cells like basal lamina, pericytes, perivascular microglia, astrocytes which are annexed to the

capillary and post capillary venules region in the CNS. BBB provides protection to CNS under normal physiologic conditions by strictly regulating the entry of plasma-born substances and immune cells into the nervous tissue. Transit changes in physiologic as well as structural characteristics of the BBB occurs during events like seizures or traumatic or ischemic CNS injuries or pathogenic infections to the brain. The endothelial cells that make up the BBB could be another center for inflammatory processes in the brain. Seizures may lead to the activation of IL-1 β system inducing neuronal cell loss and a breakdown in the BBB (Ravizza et al., 2009). This breakdown leads to leakage of BBB and the entry of albumin into the brain. Albumin entry can induce a further upregulation in inflammatory mediators and reduce the potassium and glutamate uptake of astrocytes, and neural excitability is increased (Heinemann et al., 2012). The enhancement of inflammatory reactions and the invasions of leucocytes is also initiated due to BBB leakage (Ransohoff et al., 2003). Hence a breakdown of BBB can increase neuronal excitability by enhancing inflammatory responses in the brain. Epileptic seizures can provoke inflammatory responses, which enhance calcium influx into neurons, activate glial cells to increase extracellular potassium and glutamate, and induce further inflammatory response via BBB break down. These inflammatory responses may promote neural hyperexcitability and decrease the seizure threshold. Thus, epileptic seizures and inflammatory mediators can form a positive feedback loop, reinforcing each other. In a seizure-related immune response, neurons are not the only brain cells to display an inflammatory phenotype in epileptic brain, since other brain cells also contributed to these seizure-related immune responses (Janigro, 2012). The chemokines and their receptors (CXCL12 and CXCR4; CCL2 and CCR2) are up-regulated in glia during seizures (Hartman et al., 2010), adhesion molecules (P- and E- selectin) are up-regulated in response to electrographic seizures at the luminal side of the endothelium forming the BBB (Librizzi et al., 2007) and regions characterized by a “leaky” BBB have been consistently reported in epileptic brain (Oby and Janigro, 2006).

Two distinct inflammatory processes have been linked to seizures. *Neuroinflammation* is present in epileptic brain where it exacerbates seizures or increases their frequency (Vezzani et al., 2012) and *systemic inflammation* can cause epileptiform neuronal discharge via loss of ionic *e.g.*, potassium (David et al., 2009) and neurotransmitter *e.g.*, glutamate (Heinemann et al., 2012) homeostasis. The neuroinflammation directly affects neurovascular and glial function, and the systemic inflammation are mediated or facilitated by loss of blood-brain barrier (BBB) function (Rivest, 2010). The inflammatory mechanisms involved in epileptogenesis is that during epileptic seizures there is release of cytokines from glial cells, thereby (1) increasing the influx of neuronal calcium; (2) enhancing extra neuronal glutamate concentration; (3) decreasing potassium. Hence the epileptic seizures and inflammatory mediators form a positive feedback loop, reinforcing each other.

2.1.1.2 Molecular cascades

There are various pathways identified in hippocampus during the epileptic process. These include disruption of the blood–brain barrier (BBB), the Cyclooxygenase (COX)-2 signaling pathway and related prostaglandins, classical

cytokines and their downstream targets, as well as Toll-like receptors (TLRs). In a normal brain, Cytokine receptors are present with both glia and neurons and during seizures and because microglia and astrocytes are the first cells producing cytokines, so these soluble proinflammatory cytokines mediators function as to communicate between microglia, astrocytes, and neurons (De Simoni and Imeri, 1998) The two inflammatory cytokines i.e. interleukin (IL)-1 β and tumor necrosis factor (TNF)- α , have their molecular as well as cellular actions on neuronal excitability and epilepsy . These molecules are released within the brain in specific circumstances .The action of these molecules is that they modify neural excitability both acutely and long term .The manipulation of IL-1 β and/or TNF- α level or their downstream pathways influences the neuronal excitability, seizure susceptibility, and epileptogenesis. IL-1 β have a potential role in models of febrile seizures (FS) and febrile status epileptics (SE) (Dub_et al., 2007), pilocarpine-induced SE in immature rats (Auvin et al., 2007). The IL-1 β has significant role in hippocampal seizures or status epileptics in rodents and exacerbates seizure activity. Intracerebral application of natural antagonist of IL-1-receptor (IL-1ra) investigated as powerful anticonvulsant agent which simultaneously antagonizes the effect of endogenous IL-1 β , has powerful anticonvulsant activity (Vezzani et al., 2002). So the mice with over expression of IL-1ra show significant reduction in susceptibility to the seizures (Vezzani et al., 2000), however with enhanced endogenous IL-1 β level proconvulsant effect also increases accordingly while neurotransmission by glutaminergic increases duration of seizures (Vezzani et al., 1999) so, we can say at the molecular level functional interactions exist between IL-1 β receptors and *N*-methyl-D-aspartate receptors (NMDA),co expressed by hippocampal neurons (Viviani et al., 2003). IL-1 β binding to its receptor increases NMDA receptor-mediated Ca²⁺ influx and surface expression of AMPA receptors (Beattie et al., 2002) and this cytokine : IL-1 β have excitatory effects i.e. it can inhibit glutamate reuptake by astrocytes (Ye ZC and Sontheimer, 1996) via TNF- α production,(Bezzi and Volterra, 2001) both resulting in elevated extracellular glutamate levels and hyper excitability, and consequently in hippocampal neurons, reduction in magnitude of currents mediated by γ -amino butyric acid (GABA) (Wang et al., 2000). The over expression of IL-6 in glia, increases seizure sensitivity towards glutaminergic agonists although no such correlation are found with cholinergic agonists. (Samland et al., 2003). However because over expression of IL-6 cause loss of GABA- and parvalbumin-positive neurons in the hippocampus of mice hence age-dependent neurodegeneration and behavioral seizures developed in TNF- α and IL-6 transgenic mice (Akassoglou et al., 1997) TNF- α is another inflammatory factor in epilepsy, and its expression is also up regulated in seizures (Probert et al.,1997).Microglia in the brain mainly releases TNF- α (Renno et al., 1995), and stimulate the astrocytes to release glutamate (Bezzi et al., 2001). Glutaminergic neurons are stimulated because of extracellular increase in glutamate concentration, thereby depolarizing their membrane potential. Thus post seizure production of inflammatory mediators can trigger neuronal hyperexcitability through modulations of ion channels and glutamate release in neurons and glia, respectively. After ischemic, traumatic, and excitotoxic damage in healthy brain tissue, there are rapid induction in the level of proinflammatory cytokines such as tumor necrosis factor- α (TNF α), interleukins which are initially expressing at very low level in health brain (Allan and Rothwell, 2001). Expression of messenger

RNA (mRNA) and levels of proinflammatory cytokines protein are enhanced, with an exception for IL-1 β , which is upregulated in brain of rats developing spontaneous seizures even 60 days after the induction of status epileptics while specificity in the region of onset and spread of seizure in the brain are increased by cytokines presence (Oprica et al., 2003). The status epileptics, prolonged seizures over 30 minutes, can cause neuronal death (Ravizza et al., 2005) through glutamate-mediated excitotoxicity, necrosis, and activation of apoptosis (Henshall, 2007). One to three days after status epileptics, both neuronal and astrocytic death are observed in the dentate hilus within the hippocampus (Kang et al 2006). Injured neurons and glia and their fragmented DNA are rapidly cleared by activated microglia (Streit et al., 1999). Another major factor having a specific role in epilepsy is prostaglandins (PGs) in addition to inflammatory cytokines. Prostaglandins (PGs) are major factors that stimulate inflammation processes. PGs markedly increase following seizures and contribute to epileptogenesis and reduction in seizure threshold (Viviani et al., 2007). With the robust production of PGs in the brain following seizures, an inducible type of COX is COX-2, but not the constitutively expressed COX-1, and is rapidly induced in the brain following seizures (Marcheselli and Bazan, 1996). In hippocampus region, seizures induced COX-2 partly through the pathway of NMDA receptors or in astrocytes in few days after seizure (Yamagata et al., 1993; Marcheselli and Bazan, 1996; Yamagata et al., 1993; Hirst et al., 1999). When COX-2 is ablated from the principal forebrain neurons (e.g. hippocampal pyramidal and dentate granule neurons) there is no effect on seizure onset or intensity in the pilocarpine model, whereas selective ablation of COX-2 which is limited to principal forebrain neurons was neuroprotective in the hippocampus (Toscano et al., 2008).

3.1 OXIDATIVE STRESS IN EPILEPSY

Oxidative stress is defined as an imbalance between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels (such as superoxide radical, O₂⁻; hydrogen peroxide, H₂O₂; hydroxyl radical, HO[•]; and nitric oxide, NO[•]) and the cellular antioxidant defense system (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione reductase, GR; and glutathione-S-transferase, GST) (Valko et al., 2006). Under the normal physiological conditions, ROS and/or RNS levels are fairly well regulated to perform important functions such as autophagy, chemical signaling, cell division, and mitogen activated protein kinase signaling and apoptosis (Mao and Franke, 2013). The ROS and/or RNS are tightly regulated because of its highly reactive nature and are frequently associated with neurodegeneration and mitochondrial dysfunction which is mainly observed after seizures during epileptogenesis (Patel, 2002). In oxidative stress there are alterations in ROS, RNS, and nitric oxide (NO) signaling pathways, whereby bioavailable NO is decreased and ROS and RNS production are increased (Tabima et al., 2012). The inflammatory responses induce the oxidative and nitrosative stress pathways, and subsequent mitochondrial metabolic processes generate highly reactive free radical molecules. This, ROS and RNS (for e.g. superoxide anion, hydroxyl radical, and Peroxynitrite) consist of active moieties that can react with other substrates. Both of these species are generated during the irradiation of ultraviolet (UV) light, X-rays, and

gamma rays, produced by neutrophils and macrophages during inflammation, products of reactions catalyzed by metals, present in air pollutants, and by products of reactions catalyzed by the electron carriers in the mitochondria (Valko et al., 2006). The brain contains a large number of easily oxidized fatty acids (20 : 4 and 20 : 6) and a limited antioxidant system and is highly sensitive to oxidative damage (Cardenas-Rodriguez et al., 2013). Oxidative stress is strongly implicated during seizures induced by excitotoxicity, due to mitochondrial ROS generation and is mainly associated with neuronal hyperexcitation caused by CNS diseases (Bondy, 1995). The possible mechanism in the pathogenesis of epilepsy is oxidative and nitrosative stress (Chang and Yu, 2010). During pilocarpine-induced status epileptics, hyperactivity or excitotoxicity of neuronal may induce an increase concentrations of free radical (Pazdernik et al., 2001). The increased activity of glutaminergic systems induces status epileptics and there is decrease in level of ATP due to change in redox potential, which leads to collapse in brain energy production and supply (Wasterlain et al., 1993). In seizures there is increase in release of glutamate and activation of NMDA receptor with decrease in an extracellular Ca^{2+} concentration and increase in cytosolic Ca^{2+} concentration (White et al, 2007). The effects mediated by Ca^{2+} during excessive glutamate receptor activation (excitotoxicity) lead to neuronal degeneration and overload of mitochondria with Ca^{2+} , so free radicals are generated. Overload of this type of Ca^{2+} leads to oxidative stress, cellular damage, and eventually cell death because of Ca^{2+} mediated opening of mitochondrial permeability transition (MPT) pores associated with apoptosis because of mitochondrial damage (either via mitochondrial pathway or necrosis) (Zoratti and Szabo, 1995). The phospholipase A_2 -dependent activity of Ca^{2+} mediated by glutaminergic receptors liberates arachidonic acid (AA), which generates O_2^- through its metabolism by lipoxygenases and cyclooxygenase for eicosanoid formation (Singh et al., 2003). The constant formation of $\text{NO}^\cdot$ by the glia is neurotoxic because it increases the neuronal sensitivity to this reactive species. The neurotoxic action of $\text{NO}^\cdot$ is likely caused by the formation of Peroxynitrite (ONOO^-), which is rapidly formed by the reaction of $\text{NO}^\cdot$ with O_2^- . Xanthine oxidase generates O_2^- when there is elevated intracellular Ca^{2+} concentration and energy deficiency.

3.1.1 Role of different factors in affecting oxidative stress.

3.1.1.1 Mitochondrial dysfunction

Because of abundant mitochondria, demand of high aerobic metabolism as well as great load of iron in the brain the susceptibility of cells of brain towards oxidative damage is enhanced greatly (Halliwell, 1992). Reason behind such enormous metabolic demand is because of requirement of large amounts of ATP for neurotransmission as well as maintaining of ionic gradients across cell membranes in the neurons which are highly differentiated in the nature. So performance of neurons critically depend upon mitochondrial function and oxygen supply because generation of major portion of neuronal ATP are via oxidative metabolism (Kann and Kovács, 2007). The mitochondria have critical functions which influence neuronal excitability, excitotoxicity, including the production of adenosine triphosphate (ATP), fatty acid oxidation, apoptosis and necrosis control, amino acid cycle regulation, biosynthesis of

neurotransmitters, and regulating the homeostasis of cytosolic calcium. Mitochondria are the main site of ROS production and are therefore extremely vulnerable to oxidative damage (Waldbaum et al., 2010). ROS formation contribute mechanically to chronic epilepsy. Prolonged seizures produce sufficient cellular superoxide (O_2^-), principle byproduct of respiration, so initiation of cascade of events by increased firing from neurons, excessive release of glutamate, activation of N-methyl-D-aspartate (NMDA) receptor, influx of cytosolic and mitochondrial calcium, and increased ATP consumption so endogenous antioxidant defenses of mitochondria are overwhelmed resulting mitochondrial damage (Liang et al., 2000; Turens et al., 1982). After status epileptics, level of endogenous aconitase (marker of O_2) start to inactivate in the rat hippocampus (Liang et al., 2000) and its one of most vulnerable area CA3 reported maximal inactivation. After status epileptics the electron transport chain complexes (1, 3, and 4) become dysfunctional (Gao et al., 2007) and there is reduction of mitochondrial N-acetyl aspartate (a metabolite synthesized from aspartate and acetyl-coenzyme A) in hippocampus (Moffett et al., 2007). And also a rise in mitochondrial H_2O_2 production, lipid peroxidation (increased malondialdehyde, MDA, and thiobarbituric acid, TBA), and mitochondrial DNA (mtDNA) damage after a seizure (Kutluhan et al., 2009). An adaptive increase of mtDNA repair occurs immediately after ROS increase induced by acute SE. However, chronic increase in ROS production is accompanied by failure in the induction of mtDNA repair (Jarrett et al., 2008). Damage to mtDNA and abnormal mitochondrial H_2O_2 production has been observed in the hippocampus of rats three months after Status epileptics. After lithium-pilocarpine induced SE, the oxidative stress (GSH) markers and specific markers of redox status in the mitochondrion (coenzyme A) is decreased in the hippocampus and become permanently damaged during epileptogenesis and chronic epilepsy even when H_2O_2 production and mtDNA damage return to control levels (Waldbaum and Patel, 2010). Neurons need large amount of ATP as they are highly differentiated cells and the mitochondrial oxidative phosphorylation is main source of ATP for neurons. Mitochondrion has a role in the homeostasis of intracellular calcium and its dysfunction may strongly affect the neuronal excitability and synaptic transmission (Patel, 2004) thus increases susceptibility to epileptic seizures (Kudin et al., 2009). So, mitochondrial dysfunction caused by the production of free radicals may increase susceptibility to seizures (Patel, 2004).

3.1.1.2 Impairment of antioxidant systems

During normal cellular metabolism various molecules are generated like ROS, including superoxide radical hydrogen peroxide hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$). Physiological levels of ROS can be scavenged by enzymatic [e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR), and peroxiredoxins (Prxs)] and non-enzymatic [e.g., vitamin C, vitamin E, and reduced form of glutathione (GSH)] antioxidant defense systems. However, excessive ROS levels due to increased ROS production, decreased antioxidant defense ability, or both leads to oxidative stress (Winyard et al., 2005). Seizure generation is related to the homeostatic imbalance of antioxidants and oxidants. Impairment of endogenous antioxidant factors against oxidative stress is involved in seizure generation. Superoxide radicals are highly reactive and can initiate

pathological oxidative metabolism, leading to the oxidation of macromolecules such as DNA, lipids, and proteins. The nervous system contains antioxidant enzymes, including SOD and GPx, that are expressed in higher quantities than CAT (Shivakumar et al., 1991). This spectrum of enzymatic defenses suggests that the brain may efficiently metabolize superoxide, but may have difficulty in eliminating the hydrogen peroxide produced by this reaction (i.e., superoxide dismutation). Hydrogen peroxide accumulation is of major concern, as the brain contains large quantities of iron and copper, which may catalyze the formation of hydroxyl radicals that can induce lipid peroxidation (Oubidar et al., 1996). Enhanced hydrogen peroxide, in turn, is reduced to water by peroxidases, mostly GPx (and Prx) in the brain. GPx levels in neuronal tissue appear to be relatively low for the prevention of peroxide insults. Furthermore, the neuronal cell membrane contains high levels of polyunsaturated fatty acids (Benatti et al., 2004). The brain is more vulnerable to injury by lipid peroxidation products than other tissues (Naffah-Mazzacoratti et al., 2001). The lipid peroxidation is an irreversible neuronal damage of cell membrane phospholipid and a possible mechanism of epileptic activity (Pazdernik et al., 2001).

4.1 CONCLUSION:

The literature clearly indicates that Inflammation and oxidative stress are mediators of acute and chronic epilepsies. The disruptions of the blood–brain barrier (BBB), the cyclooxygenase (COX)-2 signaling pathway and related prostaglandins, classical cytokines and their downstream targets, as well as Toll-like receptors (TLRs) are various pathways identified in hippocampus during the epileptic process. These inflammatory responses promote neural hyperexcitability and decrease the seizure threshold. Seizures also dysregulate the tightly regulated mechanisms for ROS and/or RNS resulting mitochondrial dysfunction and mitochondrial ROS generation culminating in neuronal hyperexcitation and neurodegeneration and bring about drastic decrease in cellular anti-oxidant defense mechanisms e.g SOD, CAT, GPx, GR, GST e.t.c resulting reinforcing oxidative stress and neuronal cell death.

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413