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Hypothermia mitigates neurochemical alterations in rat's cerebral cortex during status epilepticus induced by pilocarpine

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Abstract. Status epilepticus (SE) is a prolonged seizure activity associated with mortality and morbidity. SE is characterized by changes in neurotransmitter systems and oxidative stress that facilitate cellular damage. These alterations represent the neurochemical mechanisms underlying the initiation and progression of seizure activity and co-existing morbidity. In the present study, amino acid levels (glutamine, glutamate, GABA, aspartate, glycine and taurine) and oxidative stress parameters malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide NO) were determined in the cerebral cortex during SE induced by pilocarpine in rats. The study has also evaluated the effects of hypothermia, as a physical non-invasive tool, on neurotransmitters and oxidative stress alterations. The results obtained revealed that there are significant increases in glutamate, GABA, glycine and taurine and NO in the cortex of pilocarpinzed rats. Hypothermia pretreatment mitigated most of the alterations induced by pilocarpine and significantly decreased GABA concentration. These findings emphasize the involvement of extrahippocampal amino acid neurotransmitters in pilocarpine-induced SE and the ameliorative role played by hypothermia.

Key words: Amino acids — Oxidative stress — Epilepsy — Status epilepticus — Hypothermia

Introduction

The pilocarpine model of temporal lobe epilepsy (TLE) in animals can reproduce behavioral, electrical and chemical characteristics observed in humans (Morimoto et al. 2004). Injection of pilocarpine (muscarinic receptor agonist) induces a status epilepticus (SE) that is characterized by tonic-clonic generalized seizures. After several hours of SE, animals go into a seizure-free period (latent period). Then, the animals display spontaneous recurrent seizures (SRSs) that characterize the chronic epileptic condition (Leite et al. 1990; Cavalheiro et al. 1991).

Although studies show that cholinergic routes were activated by pilocarpine administration, several neurotransmitter systems were involved in initiation and maintenance of convulsions during establishment of this model (Turski et al. 1983). Several studies clearly indicate the importance of amino acids in epileptic phenomenon (Smolders et al. 1987; Fritschy et al. 1999; Sarkis et al. 2000; Cross et al. 2004). Disturbances in the brain metabolism of some amino acids especially glutamate and GABA may lead to seizures (Szyndler et al. 2008). In addition, the glutamate release by activated microglia induces excitotoxicity and may contribute to neurodegeneration in numerous neurological diseases, including epilepsy (Takeuchi et al. 2008). It is known that GABA and glutamate can exert anti- and proconvulsive effects, respectively, in seizures and SE induced by pilocarpine (Treiman 1995; Solberg and Belkin 1997). However, little is known about the alterations in amino acid contents during pilocarpine-induced SE.

In normal conditions, there is a steady-state balance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and their destruction by the cellular antioxidant system. Oxidative stress can damage the organism if the physiological balance between oxidants and anti-oxidants is disrupted. Studies have been conducted during SE induced by pilocarpine to indicate whether lipid peroxidation, nitrite concentration and GSH are involved in the pathophysiology of SE in this model (Junior et al. 2009; Aguiar et al. 2012)

The extent of damage due to neuronal insult is essentially influenced by brain temperature (Dietrich 1992). It has been shown that a moderate reduction of brain temperature by about 2–5°C has a protective effect on histopathological

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damage (Chopp et al. 1991; Chen et al. 1992a, 1992b; Morikawa et al. 1992) and behavioral deficits (Green et al. 1992). Several reports have shown that hypothermia can prevent or mitigate the neuronal insults through reduction in cerebral metabolism (Polderman et al. 2008), inhibition of the activation of caspase enzymes (Xu et al. 2002), prevention of mitochondrial dysfunction (Gong et al. 2012), deceasing the overload of excitatory neurotransmitters (Irazuzta et al. 1999; Hachimi-Idrissi et al. 2014) or modifying the severe disorders of intracellular ion concentrations (Ding et al. 2000) occurring due to neuronal insult. However, the anticonvulsant mechanism by which hypothermia works is not fully understood. Brain hypothermia and its underlying neuroprotective mechanisms should be elucidated in order to improve therapeutic outcomes.

The previous reports have mostly focused on the hippocampus as an origin structure of TLE. However, the present work aimed to investigate the alterations in amino acid neurotransmitter levels and oxidative stress in the cerebral cortex during status epilepticus induced by pilocarpine in rats. Furthermore, the possible effects of hypothermia on these neurochemical alterations were investigated.

Materials and Methods

Experimental animals

The experimental animals used in the present study were adult male Wistar albino rats weighing 200–250 g. The animals were purchased from the animal house of the National Research Center and were given food and water *ad libitum*. They were maintained under fixed appropriate conditions of housing and handling. All experiments were carried out in accordance with research protocols established by the animal care committee of the National Research Center, Egypt.

Drugs and chemicals

Pilocarpine was obtained from Macfarlan Smith Ltd. (Edinburgh). It was dissolved in saline. Atropine sulphate was obtained from Boehringer Ingelheim (Germany).

Design of experiment

Rats were divided into three groups. Group 1 consisted of control animals (6 animals) which injected only saline and serves as control animals for both the other two groups. Group 2 – pilocarpinized animals (7 animals), which were injected intraperitoneally with a single dose of pilocarpine (380 mg/kg) according to Turski et al. (1983). Atropine sulphate was injected subcutaneously at a dose of 5 mg/kg, 30 min before the induction of epilepsy, to prevent peripheral muscarinic stimulation (Williams and Jope 1994). Rats of the third group (8 animals) were exposed to hypothermia for 30 min before pilocarpine administration. The core temperature of the animals was measured by inserting a thermometer's probe about 4 cm into the animal's rectum through the anal opening. Each animal was placed into a plastic cylinder suitable for its size and has a closable wide opening and a narrow exit for the animal's breathing. The animal was placed into a refrigerator's freezing box (ambient temperature: –5 ± 1°C). Immediately after bringing the animal out of the freezing box, the core temperature was re-measured (core temperature = 26 ± 2°C). All the animals were sacrificed after 1 h of pilcarpine injection. After decapitation, the brain was transferred rapidly to an ice-cold Petri dish where it was dissected to remove the cortex. The cortex of each animal was divided into two equal halves. The left half of each brain area was homogenized in 5% w/v 20 mM phosphate buffer, pH 7.6 and used for the analysis of oxidative stress parameters. The right half was homogenized in 75% ethyl alcohol and used for the determination of amino acid levels. The brain samples were weighed and kept at –53°C until analyzed.

A separate group was used to study the dissociation of the brain temperature from the systemic temperature. The core temperature of each animal was recorded before exposure to hypothermia as described. Immediately after exposure to hypothermia for 30 min, the core temperature was recorded. The rats were decapitated, the scalp of each rat was removed and the temperature at the surface of the cerebral cortex was measured. The mean core temperature of the animals before cooling was 35.2 ± 0.4°C and after cooling was 26.6 ± 0.6°C. The temperature at the surface of the cerebral cortex was 28.3 ± 0.6°C.

Determination of nitric oxide level and lipid peroxidation

The assay of nitric oxide (NO) was carried out using Biodiagnostic kit No. NO 25 33 (Biodiagnostic Co., Egypt). This method is based on the spectrophotometric method of Montgomery and Dymock (1961) which is based on the measurement of endogenous nitrite concentration as an indicator of nitric oxide production. It depends on the addition of Griess Reagents which convert nitrite into a deep purple azo compound whose absorbance is read at 540 nm in a Helios Alpha Thermospectronic (UVA 111615, England).

Lipid peroxidation (LP) was determined by measuring the level of thiobarbituric reactive species using the method of Ruiz-Larrea et al. (1994) in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex having peak absorbance at 532 nm.

Determination of reduced glutathione level

The assay of reduced glutathione (GSH) levels was performed using Biodiagnostic kit No. GR 25 11 (Biodiagnostic Co., Egypt) based on the spectrophotometric method of Beutler et al. (1963). It depends on the reduction of 5,5'-dithiobis 2-nitrobenzoic acid with glutathione to produce a yellow color whose absorbance is measured at 405 nm.

Determination of amino acids concentration

The quantitative determination of the amino acids (glutamate, aspartate, glutamine, GABA, glycine and taurine) was carried out by using the high performance liquid chromatography (HPLC) method employed by Marquez et al. (1986). The HPLC system consisted of a Wellchrom Mini-star K-501 pump (Knauer, Germany), a column thermostat 5–85°C with injector equipped with a 20 µl loop (Knauer, Germany), a luna 5u C-18 reversed phase column (5 µm particle size, 150 × 4.6 mm I.D.) from phenomenex, USA, a Wellchrom spectrophotometer K-2600 with variable wavelength (Knauer, Germany) and a chromatography workstation (Eurochrom 2000). The mobile phase consisted of 50/50 (v/v), methanol/water containing 0.6% glacial acetic acid and 0.008% triethylamine. The concentrations of the amino acids were expressed as µmol/g fresh tissue.

Statistical analysis

The data were expressed as mean ± S.E.M. Data were analyzed by analysis of variance (ANOVA) followed by the Duncan multiple range test when the F test was significant ($p < 0.05$). All analyses were performed using the Statistical Package for Social Sciences (SPSS) software in a PC-compatible computer. Percentage difference (PD) was calculated by the following equation: [(Treated – Control) / Control] * 100

Results

Animals injected by pilocarpine became, after about 30 minutes, hypoactive. Then, they showed typical behavioral manifestations associated with this epileptic model, such as facial automatisms, salivation, eye-blinking, twitching of vibrissae and yawing. After about 40 minutes, generalized convulsions appeared on animals. Hypothermic animals showed no clear manifestation of the symptoms of SE. They were slightly sedated and exhibited only shivering and tremors after pilocarpine injection.

Table 1 shows the effects of pilocarpine-induced SE on amino acid levels in the cortex of male albino rats. There were significant increases of 20, 15, 16 and 14% in glutamate, GABA, glycine and taurine above the control levels, respectively. Non-significant increases of 2 and 7% were recorded in glutamine and aspartate above the control levels, respectively.

As shown in Table 2, a non-significant decrease in MDA of 13% was obtained in the cerebral cortex of pilocarpine-induced SE and a non-significant increase of 15% was obtained in the case of GSH from the control values. A significant increase of 44% was recorded in NO concentration above the control value.

Application of hypothermia before induction of SE in rats has mitigated the alterations in amino acid levels after pilocarpine administration as shown in Table.1. Glutamine, glutamate, aspartate, glycine and taurine have recorded non-significant changes of –8, –5, 4, 5 and –3% from the control levels, respectively. However, GABA recorded a significant decrease of 20% from control level during SE.

Hypothermia has also decreased, non-significantly, the differences of MDA and GSH and prevented the significant increase of NO level obtained after pilocarpine-induced SE in rat's cortex (Table 2). Percentage differences of –5, 8, and 17% were obtained for MDA, GSH and NO from control values, respectively.

Table 1. Amino acids levels (µmol/g tissue) in the rat's cortex of pilocarpine-induced SE in control, pilocarpinzed and hypothermic pretreated animals

	Control	Pilocarpine	PD	Hypothermia	PD
Glutamine	4.64 ± 0.15	4.75 ± 0.26	2.37	4.25 ± 0.12	–8.41
Glutamate	12.09 ± 0.25	14.53 ± 1.12*	20.18	11.45 ± 0.19	–5.29
Aspartate	8.94 ± 0.39	9.56 ± 0.28	6.94	9.34 ± 0.23	4.47
GABA	2.03 ± 0.04	2.34 ± 0.06*	15.27	1.62 ± 0.04*,#	–20.19
Glycine	1.18 ± 0.04	1.37 ± 0.05*	16.10	1.24 ± 0.03	5.08
Taurine	6.16 ± 0.12	7.01 ± 0.46*	13.79	6.00 ± 0.17	–2.59

Data are mean ± S.E.M. * $p < 0.05$ *vs.* control; # $p < 0.05$ *vs.* pilocarpine-treated animals. PD, % difference with respect to control values; SE, status epilepticus.

Table 2. Oxidative stress markers values in the rat's cortex of pilocarpine-induced SE in control, pilocarpinzed and hypothermic pretreated animals

	Control	Pilocarpine	PD	Hypothermia	PD
MDA (nmol/g)	4.72 ± 0.35	4.11 ± 0.23	12.92	4.49 ± 0.2	–4.87
GSH (mmol/g)	0.62 ± 0.04	0.71 ± 0.06	14.52	0.67 ± 0.06	8.06
NO (μmol/g)	1.36 ± 0.13	1.96 ± 0.13*	44.11	1.59 ± 0.11	16.91

Data are mean ± S.E.M. * $p < 0.05$ *vs.* control. PD, % difference with respect to control values; SE, status epilepticus; MDA, malondialdehyde; GSH, reduced glutathione; NO, nitric oxide.

Discussion

Temporal lobe epilepsy (TLE) is the most common drug-resistant type of adult epilepsy, which is characterized by hippocampal sclerosis leading to reorganization of neuronal networks. Currently, it is one of the most frequently used models to study the mechanisms of epileptogenesis and to test new compounds for epilepsy treatment (Curia et al. 2008).

In the present work, pilocarpine injection resulted in a significant increase in glutamate in the rat's cortex. Elevation of glutamate concentration has been observed in patients with complex partial seizures subjected to epilepsy surgery (Carlson et al. 1992; During and Spencer 1993; Nomura et al. 2014). It was reported that muscarinic receptor stimulation by pilocarpine administration may lead to a long-lasting facilitation of NMDA receptor-mediated postsynaptic potentials (Markam and Segal 1990; Naylor et al. 2013) which leads to enhancement of glutamate levels in different brain regions. A pronounced glutamate release followed by activation of NMDA, kainate and metabotropic receptors has been associated with glutamate excitotoxicity and neuronal death in several structures during SE (Millan et al. 1993; Szczurowska and Mares 2013).

The significant increase in NO recorded in this study in pilocarpinzed rats could be attributed to the significant increase in glutamate, as the production of NO is linked to the activation of NMDA receptors (Schuman and Madison 1994). Di Maio et al. (2011) reported that the Ca^{2+} uptake by mitochondria in combination with NO production triggers cell death.

The significant increase in the excitatory amino acid glutamate in the cortex of pilocarpinzed rats indicates a state of hyperexcitability that could be responsible for the secondary cortical generalization (originated in hippocampus) seen in this model of epilepsy. Furthermore, the present recorded increase in glycine content in the cortex may enhance the state of excitation as glycine potentiates NMDA receptors (Larson and Beitz 1988). Supporting this present finding, reports showed elevations of aspartate, glycine, and glutamate concentrations during the onset of seizures in the cerebral cortex of epileptic patients (Carlson et al. 1992; Sherwin 1999). Furthermore, Engstrom et al. (2001) reported elevations of aspartate and glutamate levels in the brain tissue of epileptic rats.

Glutamate is transformed to glutamine in the glial cells *via* the specific enzyme glutamine synthetase (GS) (Norenberg and Martinez-Hernande 1979; Brookes 2000) and released to be taken by neurons and transported to the synaptic terminal for the subsequent re-synthesis of glutamate (glutamatergic neurons) and GABA (GABAergic neurons). The present data recorded moderate increase in glutamine level in the rat's cerebral cortex during SE. This may indicate that the glutamate-glutamine cycle is not compromised in rat's cerebral cortex during SE. It may also indicate an early increase in the glutamate-glutamine cycle which ultimately leads to the enhancement of the inhibitory system as a compensatory response.

When activity levels of neuronal networks are altered, cellular excitability and synaptic strength within the network are adjusted in a direction that appears to oppose the alteration in activity (Burrone et al. 2002; Wierenga et al. 2005). In the present study, the significant increase in GABA and taurine level in the cortex during this early stage of SE (1 hour) seems to be of that kind of adjustment that occurred in the cortical network in response to the increase in glutamate level. Additionally, a significant increase in glutamic acid decarboxylase (GAD), the enzyme responsible for the conversion of glutamate to GABA, has been reported in several models of epilepsy (Feldblum and Ackermann 1990; Baran et al. 2004). The increase in GAD activity may underlie the cortical rise in GABA level recorded in the present study.

Taurine is an inhibitory amino acid acting as an osmoregulator and neuroromodulator in the brain, with neuroprotective properties. The NMDA-evoked release of taurine both *in vivo* and *in vitro* has been well documented. For instance *in vitro*, NMDA has evoked taurine release from mouse cerebral cortical slices (Saransaari and Oja 1991). The significant increase in taurine during SE in the present study could be associated with the increase in the activity of the NMDA receptors obtained after pilocarpine injection. Taurine exerts its protective function against glutamate-induced neuronal excitotoxicity by counteracting the glutamate-induced increase of free intracellular calcium

and by preventing the glutamate-induced membrane depolarization (Wu et al. 2005).

Neuropathological and electrophysiological data have shown that although limbic structures are involved in the pathogenesis of TLE, in most cases, limbic-originated seizures frequently spread to extrahippocampal areas (Silva et al. 2002; Xia et al. 2009). Abnormal intra-cortical inhibition and facilitation was observed in adult TLE patients during extra-operative cortical stimulation, suggesting a remote effect of epileptic activity onto the cortex that leads to an alteration in local circuits (Werhahn et al. 2000). Additionally, acute pilocarpine-induced seizures typically become generalized much more rapidly, which suggests that this model has a strong cortical component during the induction period that could trigger seizure-dependent damage in cortex. Recently, Mathew et al. (2012) showed that alteration in GABA receptors in the cerebral cortex of pilocarpine-induced epilepsy in rat comprise an important role in seizure initiation and memory deficits associated with epilepsy.

The induction of hypothermia in rats prior to injection of pilocarpine in the present study has greatly alleviated the typical behavioral symptoms characterizing this animal model. This is in line with the study of Yu et al. (2011) who found that pretreatment of animals with hypothermia protected the animal from the deleterious effects of SE. Several other reports demonstrated the ameliorative and therapeutic potential of hypothermia particularly in refractory SE (Liu et al. 1993; Maeda et al. 1999; Rothman and Yang 2003; Schmitt et al. 2006; Bagic et al. 2008). Recently, Bo et al. (2014) reported the protective effect of mild hypothermia pretreatment against injury to rat cortical neurons by glutamate *in vitro*.

Hypothermia in the present study has preserved glutamate and glycine concentrations from change with respect to control level after pilocarpine injection. These findings emphasize the capacity of hypothermia to modulate excitotoxic transmission. Studies have provided evidence that hypothermia reduces extracellular concentrations of both glutamate and glycine (Baker et al. 1991; Kvrivishvili 2002). Yu et al. (2012) reported that hypothermia might exert its protective effects against pilocarpine-induced SE by regulating glutamate receptor expression. Friedman et al. (2001) reported that glutamatergic receptors AMPA and NMDA are modulated by hypothermia. Therefore, this modulating effect of hypothermia on the excitatory transmitters which prevents their excessive release could eventually lead to a reduction in the calcium overload and Ca^{2+}- mediated neuronal damage.

In the present study, hypothermia prevented the increase in the concentration of NO induced by pilocarpine injection. It has been reported that NO is a highly reactive radical playing a major role in neurotoxicity during seizures (Dawson et al. 1991; Ryan et al. 2014). This hypothermic-induced inhibition of NO generation in the cerebral cortex during SE may be a possible mechanism of neuroprotective effects of hypothermia. Van Hemelrijck et al. (2005) have reported that mild hypothermia can inhibit the expression of NOS in cortex and reduce NO and its metabolites thereby playing a neuroprotective role.

Hypothermia induces slowing of cerebral metabolism (Polderman et al. 2008) leading to reduced glucose concentration (Nomura et al. 2014). Glucose is the main substrate for most of the amino acids including GABA and glutamate (Schousboe et al. 2007). Therefore, the amelioration of glutamate increase and the significant decrease in GABA recorded in hypothermic animals after pilocarpine injection in the present study could be attributed to the reduction in cerebral glucose metabolism. Kaibara et al. (1999) reported that hypothermia induces a depression in the flux of the tricarboxylic acid cycle (TCA) which is responsible mainly for production of GABA and glutamate in glial cells.

Taking together, the neurochemical changes taking place in the cortex of pilocarpine injected animals and hypothermic treated animals with respect to control indicated that pilocarpine as a cholinergic drug causes imbalance in excitatory/inhibitory neurotransmission balance that in turns leads to the behaviorally symptoms of status epilepticus. It indicates also, that neurochemical state in hypothermic animals could interpret the lack of status epilepticus symptoms in these animals.

In conclusion, the present study revealed that the marked variations in amino acid neurotransmission and NO in the cortex of the experimental animal model of epilepsy during SE may underlie the propagation and progression of epileptogensis. Furthermore, hypothermic pretreatment modulated this cortical hyperexcitability state after epilepsy induction by pilocarpine and this was reflected in the reduction of epileptic manifestations in these animals. Further studies are needed to elucidate the safety of hypothermic application at the onset of SE.

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