OXIDATIVE STRESS AFTER LITHIUM-PILOCARPINE INDUCED STATUS EPILEPTICUS IN MICE BRAIN

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Abstract

The imbalance between oxidants and the antioxidant defense system in Li-pilocarpine induced status epilepticus (SE) was studied by measuring lipid peroxidation, glutathione (GSH), total thioles (TSH) and the activities of superoxide dismutase (SOD) and catalase in the cerebral cortex of mice. SE was induced by treating the animals with LiCl (3 meq/kg, i.p.) followed 21 h later by pilocarpine challenge (30 mg/kg, i.p.), while control animals were administered normal saline. A significant increase (37.5%) in lipid peroxidation was observed in brain after the induction of SE. Concomitantly, the GSH and TSH levels were found to decrease by 16.7 and 19.1% respectively in brain of animals treated with Li-pilocarpine. This was accompanied by a significant increase in activities of catalase and SOD (40.8% and 81.4%, respectively) as compared to the control group. These results show evidence of neuronal damage in the cerebral cortex in terms of increase in lipid peroxidation and a decrease in levels of protein and non-protein thiol groups. Our studies clearly suggest that oxidant-antioxidant balance is disturbed in status epilepticus.

Key words: Status Epilepticus, Lipid peroxidation, Glutathione, Catalase, Superoxide Dismutase.

Introduction

Status epilepticus (SE) is a medical emergency with an associated mortality of 10 to 12% (1). Lithium-pilocarpine (Li-pilocarpine) is a widely used animal model of epilepsy to investigate the development of neuropathology in SE. Despite numerous studies indicating the importance of neurochemical alterations in the epileptic phenomenon, the biochemical mechanisms involved are not completely understood (2). Oxidative injury is an important factor in pathogenesis of many acute and chronic diseases of the central nervous system (3). Oxidative stress is a situation of the imbalance between the production of oxidants and the respective defense system of an organism. Oxidants, such as reactive oxygen species and nitrogen species can damage biomolecules by chain reactions in which one radical can induce the oxidation of a large number of substrate molecules (4). Free radicals can react with lipid bilayer of cell membrane leading to peroxidation of polyunsaturated fatty acids and release of toxic by-products that may react with proteins leading to enzyme inactivation and disturbances of cellular function (4, 5). This is particularly true in brain as it contains large amounts of oxidizable lipids and has low antioxidant capacity as compared to other tissues.

In the nervous system, the phenomenon known as excitotoxicity has been related to over-production of free radicals (6). Neuronal hyperactivity and/or excitotoxicity may be involved in increase in free radical production during pilocarpine-induced SE (7). Free radical generation can induce seizure activity by direct inactivation of glutamine synthase thereby permitting an abnormal build up of excitatory neurotransmitter glutamic acid (8). Involvement of oxidative stress in SE is supported by a study by Costa-Lotufo et al., (2002) which demonstrated attenuation of pilocarpine induced seizures by melatonin (9). These findings suggest the involvement of ROS in the epileptic cell damage and free radical scavenging may prevent SE-induced cell loss. The aim of the study was to investigate the effect of SE on malondialdehyde (MDA) levels as marker of lipid peroxidation, and on antioxidant defense system in terms of glutathione (GSH) levels, total thioles (TSH) levels and antioxidant enzymes: superoxide dismutase (SOD), catalase activities in the cerebral cortex of mice.

Experimental Procedures

Treatment of animals

Mice (Lacca strain), weighing between 30-40 g, procured from Central Animal House of Panjab University, Chandigarh were used. The animals were housed in polypropylene cages in the departmental animal house under hygienic conditions and were fed with standard pellet diet and water ad libitum. The animals were divided into 2 groups of 6 animals each.

Control group: Animals in this group received an equal amount of normal saline (i.p.).

Li-pilocarpine treated group: Animals in this group were treated with LiCl (3 meq/kg, i.p.) followed 21 h later by pilocarpine challenge (30 mg/kg, i.p.).

Tissue preparation

After 24 h of treatment, animals in both the groups were anaesthetized with light ether and sacrificed by decapitation. Their brains were dissected on ice and cerebral cortices were removed, rinsed in ice-cold normal saline (0.9% w/v NaCl), blotted dry and weighed separately. The tissues were homogenized in 10% w/v 10 mM Tris-HCl (pH 7.4) using mechanically driven Teflon fitted Potter-Elvejhem type homogenizer for few strokes for total disruption of cells. The homogenate was centrifuged at 1000xg for 10 min at 4°C. Pellet was discarded and supernatant was again centrifuged at 12,000xg for 20 min to obtain post mitochondrial supernatant (PMS) and was used for SOD and catalase assay. Lipid peroxidation, glutathione and total thioles were assayed in the homogenates.

Lipid peroxidation

Lipid peroxidation was analyzed by measuring malondialdehyde (MDA) levels as described by Wills (10). Briefly, 0.5ml of tissue homogenate was diluted with 0.5ml Tris-HCl buffer. After 2 hours of incubation, 1.0 ml of ice cold 10% (w/v) TCA was added and, the contents were centrifuged at 800xg for 10 min. To 1.0 ml of
Table 1: Effect of Li - Pilocarpine induced SE on lipid peroxidation and thiol content in the Cerebral Cortex of Mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lipid Peroxidation (nmol MDA/mg protein)</th>
<th>Glutathione (nmol /mg protein)</th>
<th>Total Thiols (nmol /mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.45 ± 0.28</td>
<td>44.72 ± 0.92</td>
<td>104.40 ± 3.13</td>
</tr>
<tr>
<td>Li-pilocarpine</td>
<td>3.37 ± 0.14*</td>
<td>37.23 ± 1.17**</td>
<td>84.46 ± 2.77**</td>
</tr>
</tbody>
</table>

Values expressed as mean ± S.D. ( n=6)
Data was analyzed by student's t-test
* Significant in comparison to control, p < 0.01
**Significantly different as compared to control, p < 0.001

the supernatant, 1.0 ml of 0.67% (w/v) thiobarbituric acid was added and the contents were incubated at 100° C for 10 min. The samples were cooled and diluted with 1.0 ml distilled water and the absorbance was read at 532nm. The results were expressed as nmoles MDA/mg protein.

**Determination of thiols**

GSH levels in the Li-pilocarpine and control animals were analyzed according to Ellman’s method (11). 0.75 ml of 4% (w/v) sulphasalicylic acid was added to 0.75 ml of brain homogenate and centrifuged at 12000xg for 5 min. To 0.5 ml of supernatant, 4.5 ml of Ellman reagent was added and absorbance read at 412 nm after 2 minutes. For TSH determination, 0.2 ml of the homogenate was pipetted. To this 0.4 ml of Tris HCl buffer (pH 8.2) and 0.1 ml of 0.1 M DTNB were added successively in stoppered tubes. This was followed by addition of 9.3 ml of absolute methanol and samples were kept for 15 min. The contents were filtered through Whatman No. 1 filter paper and absorbance read at 412 nm. The results were expressed as nmol /mg protein.

**Antioxidant enzymes**

Catalase activity was determined according to the method of Luck (12). 3 ml H2O2 phosphate buffer (12.5 mM H2O2 in 67.0 mM Phosphate buffer pH 7.0) was pipetted directly into the cuvette. Appropriate amount of PMS was added to the buffered solution. The contents were mixed and decrease in absorbance was followed at 240 nm for 3 min. The results were expressed as nmol of H2O2 decomposed/min/ mg protein using mmolar extinction coefficient of H2O2 (71mM-1cm-1) at 240nm.

SOD activity was assessed by the method of Kono (13). Non-enzymatic reaction, serving as control consisted of 2ml NBT, 0.5 ml hydroxylamine hydrochloride and development of blue color was measured at 560 nm for 2 min. Enzymatic reaction, serving as test, consisted of 2ml NBT, 0.5 ml hydroxylamine hydrochloride, appropriate amount of enzyme source and development of blue colored complex was measured at 560 nm for 2 min. Enzyme activity was expressed as Units/ mg protein, where 1 unit of enzyme is defined as amount of enzyme inhibiting NBT reduction by 50%.

**Statistical Analysis**

All the results were expressed as mean ± S.D. The comparison of the control data with the treated data was statistically analyzed by using students “t” test to observe the validity of the data. Differences were considered statistically significant when p<0.05.

**Results and Discussion**

Lipid peroxidation and Thiols in Cerebral Cortex after Li-pilocarpine induced SE - Epilepsy and oxidative stress are thought to be closely interrelated. Our findings show that enhanced lipid peroxidation in cerebral cortex of Li-pilocarpine induced SE along with reduction in the levels of GSH and TSH. As shown in Table.1, Li-pilocarpine treatment produced a significant (37.5%) increase in the MDA concentration [p<0.01]. On the other hand, a 16.7% decrease [p<0.001] in GSH content and a 19.1% decrease [p<0.001] in TSH concentration were detected. Lipid peroxidation is an index of irreversible neuronal damage of cell membrane phospholipid and has been suggested as a possible mechanism of epileptic activity (14). NMDA receptor activation during epilepsy leads to an influx of Ca2+ into the cells causing increased intracellular calcium levels. The massive influx of Ca2+ through a series of as yet unclear steps may activate phospholipase activity leading to production of free radicals from the arachidonic acid pathway, eventually leads to lipid peroxidation and cell death (15).

During ROS scavenging, GSH reduction and glutathione disulfide production occur. When the balance between ROS formation and ROS elimination is functionally normal, there is GSH recovery (16). As mentioned above, we can conclude that during SE there is over-formation of free radicals and/or a deficiency of antioxidant systems, as evidenced by the augmented MDA concentration and GSH consumption as well as reduced levels of TSH, all of which characterize oxidative stress. GSH depletion plays an
important role in the neurodegeneration in the cortex during SE, GSH being involved in mechanisms responsible for eliminating oxygen free radicals during the establishment of SE (17). Additionally, the activities of antioxidant enzymes, such as glutathione peroxidase, glutathione reductase, and Cu-ZnSOD have been shown to be affected by GSH depletion (18). The cause of GSH depletion is not clear but it has been reported that the GSH content of brain cells depends strongly on the availability of precursors for GSH (19). The decreased level of TSH may be due to decreased GSH content and protein content. The decreased TSH content may implement its effects by changing protein conformation and by altering the thiol groups present in enzyme active sites i.e. decreased TSH/GSSP serves as a signal for redox-sensitive reactions such as protein synthesis, detoxification, cell division, and regulation of intracellular signal transduction to be targeted (20). In brief, subtle changes in intracellular thiol-disulfide status have an important bearing on the molecular events associated with cellular response to the stress.

Superoxide Dismutase and Catalase activities in Cerebral Cortex after Li-pilocarpine induced SE- Fig 1 shows SOD and catalase activities in the cerebral cortex after seizures and SE induced by Li-pilocarpine. Catalase activity was markedly (40.8%) increased [p < 0.001] in this model compared with the corresponding values in free radical formation is accompanied by an immediate compensatory increase in catalase and SOD activities, which may be a long-term compensatory mechanism including activity modulation of enzymes (21). In addition, in the normal physiological state, changes in the neuronal activity are accompanied by alterations in the metabolic rate (oxygen and energy metabolism) (1, 2), which induce modifications in the cerebral blood flow (22). In the pathological states blood flow may not occur in the same way. There is clinical and experimental evidence of alterations in oxygen levels because of reduced oxygen availability after status epilepticus (22). Thus, augmented activities of both catalase and SOD may be related to increased metabolic demand.

The results obtained from the present work indicate clearly that enhanced oxidative stress has an important role to play in mechanisms leading to neurodegeneration associated with SE as evidenced by its effects on lipid peroxidation, GSH content and total thiols concentration. Furthermore, the results suggest that GSH along with catalase and SOD activity play an antioxidant role in the cortex during SE. GSH as well as catalase and SOD activity are involved in mechanisms responsible for eliminating oxygen free radicals during the establishment of SE in the brain. The results of our studies and the observations of other authors suggest that oxidants-antioxidants balance is disturbed in SE. The information might unravel not only mechanistic questions but also potentially therapeutic ones. Further studies need to be carried out to ascertain whether ROS are involved in the pathogenesis of status epilepticus.
References


