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Seizure-induced oxidative stress in rat brain regions: blockade by nNOS inhibition

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Abstract

Free radicals have been implicated in the pathogenesis of various neurological disorders including epilepsy. Experimental seizures are often accompanied by the generation of free radicals that cause lipid peroxidation (LPO), which may subsequently cause neurodegeneration observed in certain types of human epilepsy. We recently reported a trigger role for nitric oxide (NO) derived by activation of neuronal isoform of nitric oxide synthase (nNOS) and that the action of conventional antiepileptic drugs (AEDs) was potentiated by inhibition of nNOS. In the present study, we extend our observations to understand the significance of blockade of the nNOS pathway on seizure-induced oxidative stress. Increased NO and LPO levels was observed at the time that corresponded to the onset of generalized seizures in rat brain regions following administration of $GABA_A$ receptor antagonist, picrotoxin (PCT). Treatment with the selective nNOS inhibitor, 7-nitroindazole (7-NI), decreased NO and LPO levels. The AEDs, diazepam and phenobarbitone also prevented seizure-induced increase in NO and LPO levels. Seizures resulted in a significant increase in the activity of antioxidant enzymes, superoxide dismutase in the frontal cortex and hippocampus. On the other hand, the activity of glutathione peroxidase was decreased in the hippocampus and midbrain. Whereas treatment with 7-NI could minimize the effects of PCT, the AEDs per se did not have any significant impact on the activity of the antioxidant enzymes, though co-treatment with 7-NI and AEDs could significantly decrease seizure-induced alterations in antioxidant enzyme activities. These observations suggest that the AEDs may not have a significant role in modulating the activities of antioxidant enzymes and that their ability to decrease LPO is realized more likely by their ability to prevent free radical formation. In conclusion, the present study demonstrates that NO contributes to LPO observed following seizures induced by PCT. The study also provides evidence for the ability of the AEDs to inhibit seizure-induced increase in LPO levels, the effect being enhanced by co-treatment with 7-NI suggesting that 7-NI and the AEDs together could prevent the neurotoxic cascade induced by oxidative stress.

Keywords: Nitric oxide; Seizures; Oxidative stress; Antiepileptic drugs

1. Introduction

Growing data from experimental models and human brain suggest that oxidative stress and injury may play an important role in pathophysiology following acute neurological insults such as stroke and seizures. Membrane lipid derangements, including lipid peroxidation (LPO), have been reported to contribute significantly to paroxysmal membrane malfunction during epileptogenesis; and enhanced free radical production and oxidative lipid damage have been demonstrated during seizures and seizure-mediated neuronal injury (Frantseva et al., 2000; Patel et al., 2001). Further support for a role of free radicals in seizures comes from the successful use of exogenously administered antioxidants in protecting the brain against seizure-induced brain damage (Lapin et al., 1998; Murashima et al., 1998; Ueda et al., 1997).

While the role of nitric oxide (NO) in seizures remains a matter of controversy with both pro- and anticonvulsant

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properties being attributed to the molecule depending upon the experimental model used (Kirkby et al., 1996), several lines of evidence suggest that NO produced by the activation of neuronal nitric oxide synthase (nNOS) triggers seizures (Rajasekaran et al., 2003; Murashima et al., 2002; Raeveskii et al., 1998).

The interaction between NO and free radicals produced during seizures could have potential consequences on the outcome of seizure-induced neuronal injury, since NO may cause neuronal damage in cooperation with other reactive oxygen species. Furthermore, the $GABA_A$ receptors, which are principal targets of anticonvulsant drug actions, are highly sensitive to oxidative stress. Sah et al. (2002) have reported that free radicals could compromise with $GABA_A$-mediated neuronal inhibition via interaction with pre- and post-synaptic sites, ultimately contributing to neuronal damage. Furthermore, a reduction in synaptosomal GABA release and uptake as a result of LPO has been reported by Haughey et al. (1999). In light of our recent observation that conventional antiepileptic drugs (AEDs) exert their anticonvulsant effects partly by inhibiting nNOS activation (Rajasekaran et al., 2003), we wanted to examine if nNOS inhibition may also relate to possible antioxidant actions. Hence, in the present study, we extend our earlier observations to investigate the influence of the effective anticonvulsant doses of nNOS inhibitor, 7-nitroindazole (7-NI) and AEDs on seizure-induced alterations in LPO and the activity of the important antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx) in rat brain regions following seizures induced by PCT. The results of this study suggests that blockade of nNOS pathway may also involve antioxidant actions.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (150–170 g) were used throughout the study. The animals were housed in groups of 3–4 per cage and had free access to food and water under controlled temperature (26–28 °C) and light (12:12-h day/night cycle). Food was withdrawn one and half hours prior to commencement of the experiment. Experiments were performed in accordance with the Institutional Ethical Guidelines and Guidelines for Breeding of and Experiments on Animal as defined by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

2.2. Chemicals

PCT and 7-NI were from Sigma Chemical, St. Louis, MO, USA. Diazepam and phenobarbitone were obtained commercially (Becker and May, India).

2.3. Drug treatment

Seizures were induced by intraperitoneal (i.p.) administration of PCT (5 mg/kg) and were visually evaluated for 1 h on the basis of a behavioral scale based on the report of Shandra et al. (1996). Twelve minutes after the administration of PCT, animals begin to develop generalized seizures characterized by clonic convulsions of the whole body and seizures typically terminate by 50 min (Rajasekaran et al., 2003).

On the basis of earlier reports investigating the role of nNOS in seizures (Montecot et al., 1997; Borowicz et al., 2000; Chavko et al., 2001; Bagetta et al., 2002) as well as our observations (Rajasekaran et al., 2003) to evaluate the dose- and time-dependent effects of 7-NI treatment, 7-NI was administered to rats at a dose of 25 and 50 mg/kg (i.p.), 30 or 60 min before administration of PCT. For co-treatment studies, animals received 7-NI and a previously determined (Rajasekaran et al., 2003) minimally effective anticonvulsant dose of diazepam (0.20 mg/kg, i.p.; 10 min) or phenobarbitone (20 mg/kg, i.p. 10 min). Each experimental group consisted of 6 animals. The drugs were dissolved in saline in such a way so as to administer 0.2 ml/100 g body weight of the animal. Control animals received an appropriate volume of saline.

2.4. Measurement of NO levels

Animals were sacrificed by decapitation, 12 min after administration of PCT. This time point corresponded to the time of appearance of generalized seizures following PCT injection (Rajasekaran et al., 2003). The frontal cortex (including the striatum), hippocampus and midbrain were dissected quickly and rapidly frozen and stored in deep freezer at −80 °C until further assay. The frozen brain tissues were homogenized in 10 volumes of 50 mM Tris (pH 7.0) and centrifuged (8000 rpm, 10 min, 4 °C). Since detergents were not used, the possibility of eNOS (which is primarily membrane bound) release is remote, and the supernatant obtained is predominantly the source of nNOS. nNOS-induced NO levels were determined spectrophotometrically by observing the oxidation of oxyhemoglobin (HbO_2) by NO to methemoglobin (Hevel and Marletta, 1994). Briefly, 500 μl of the supernatant was added to reaction mixture containing 1 mM L-arginine, 1 mM $CaCl_2$, 1 μM calmodulin, 5 μM HbO_2, 100 μM NADPH and 100 mM HEPES (pH 7.5) at 37 °C and the difference in absorbance between 421 and 401 nM at the end of 5 min was recorded using a dual-wavelength spectrophotometer. To ensure against non-specific oxidation of hemoglobin affecting the experiment, HbO_2 was discarded and freshly prepared again if the ratio of its absorbance at 576 nm/540 nm became less that 1.05. Further, in situ controls by using NOS inhibitor, L-NAME (5 mM), in the reaction mixture were randomly carried out. The percentage of non-specific oxidation was found to be less than

5%. NO levels were represented as nmol HbO_2 oxidized in vitro/min/g tissue.

2.5. Measurement of LPO

LPO levels in the brain regions were assayed spectrophotometrically (532 nm) by the method of Ohkawa et al. (1979), in which the malondialdehyde release served as the index of LPO. The LPO values were represented as μmol of thiobarbituric acid reactive substances (TBARS) produced/g tissue.

2.6. Measurement of superoxide dismutase (SOD) activity

The activity of SOD was determined spectrophotometrically (480 nm) as per established procedures (Marklund and Marklund, 1974) by determining the rate of inhibition of pyrogallol auto-oxidation. The enzyme activity was expressed as units/mg protein in which one unit corresponds to the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. Protein levels in the tissue were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.7. Measurement of glutathione peroxidase (GPx) activity

This enzyme was assayed spectrophotometrically (420 nm) in the brain homogenate according to the method of Rotruck et al. (1973). GPx activity was expressed as μg of glutathione utilized/min/mg protein.

2.8. Statistical analysis

Data were analyzed statistically using one-way ANOVA followed by Tukey's multiple comparison test. Data were expressed as mean±S.E.M. of 6 (n=6) animals. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Seizure-induced alteration in NO levels in rat brain regions: effect of 7-NI and AEDs

Animals were sacrificed at the onset of generalized seizures, and they had significant (P<0.001) increases in NO level in all three brain regions examined (Fig. 1). 7-NI treatment could suppress PCT-induced increase in NO levels by up to 46% in the frontal cortex and hippocampus and by 37% in the midbrain, respectively. Similarly, the AEDs could also significantly reduce seizure-induced increase in NO levels in all brain regions. Diazepam per se decreased PCT-induced increase in NO level by 21.5% (P<0.01) in the frontal cortex and hippocampus and 18.5% in midbrain (P<0.05), respectively. Likewise, phenobarbitone decreased PCT-induced increase in nNOS activity by up to 23% in

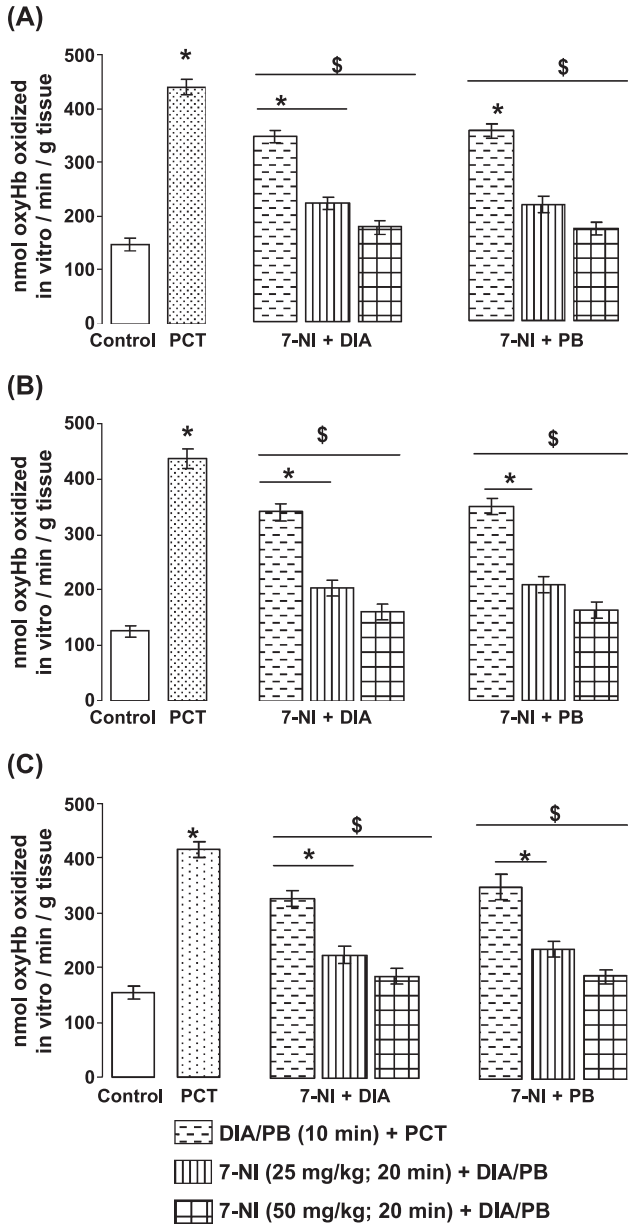

Fig. 1. Effect of co-treatment with 7-NI and AEDs on seizure-induced changes in the levels of nitric oxide in the (A) frontal cortex (B) hippocampus and (C) midbrain. Data represents mean±S.E.M. of 6 animals. *P<0.05 compared to control; $$P$<0.05 compared to PCT-treated rats (ANOVA followed by Tukey's multiple comparison test).

frontal cortex and hippocampus and 21% (P<0.05) in the midbrain, respectively.

Co-treatment with 7-NI at both doses (25 and 50 mg/kg) and time points (30 and 60 min) increased significantly the effect of the AEDs in decreasing PCT-evoked increase in NO levels in the various brain regions. Co-treatment of 7-NI increased the ability of diazepam to inhibit PCT-evoked increase in NO levels by up to 55% (P<0.001) in the frontal cortex (Fig. 1A) and hippocampus (Fig. 1B) and 50.5% in the midbrain (Fig. 1C), respectively. In phenobarbitone-treated animals, co-treat-

ment of 7-NI at the lower dose (25 mg/kg) itself could inhibit PCT-induced increase in NO levels to near normal values. 7-NI thus significantly ($P<0.001$) enhanced the action of phenobarbitone by up to 55% in the frontal cortex (Fig. 1A) and hippocampus (Fig. 1B) and 50% in the midbrain (Fig. 1C).

3.2. Seizure-induced alteration in LPO level in rat brain regions: effect of 7-NI and AEDs

PCT-induced seizures resulted in significant ($P<0.001$) elevations in LPO levels in the frontal cortex (Fig. 2A) and midbrain (Fig. 2C). Similarly, significant increase ($P<0.01$) in hippocampal LPO levels was also observed (Fig. 2B). Both 7-NI and the AEDs were able to significantly decrease seizure-induced elevation in LPO levels in the frontal cortex and hippocampus, but not the midbrain. Accordingly, in the frontal cortex, 7-NI treatment (50 mg/kg dose; 60 min time) maximally ($P<0.01$) reduced PCT-induced increase in LPO levels by 31.5%. At the same dose and treatment time, there was a 20% ($P<0.05$) decrease in seizure-induced of LPO level in the hippocampus. Both diazepam and phenobarbitone reduced seizure-induced LPO elevations only in the frontal cortex (Fig. 2A) and not the hippocampus (Fig. 2B) or midbrain (Fig. 2C). Diazepam decreased seizure-induced elevation in LPO by 17.2% in the frontal cortex ($P<0.05$), whereas phenobarbitone by 25.4% ($P<0.001$).

The effect of the AEDs in decreasing PCT-induced increase in LPO was enhanced by co-treatment with 7-NI. Co-treatment with 7-NI and diazepam or phenobarbitone significantly decreased ($P<0.001$ compared to PCT) seizure-induced LPO elevation in all brain regions (Fig. 2). In the frontal cortex, co-treatment with 7-NI (50 mg/kg; 60 min) maximally enhanced the effect of diazepam and phenobarbitone by 41.3% and 36.8%, respectively. The effects of diazepam and phenobarbitone on PCT-induced increase in hippocampal (Fig. 2B) LPO levels were further increased by 7-NI co-treatment (50 mg/kg; 60 min) by up to 21.6% and 21.8%, respectively. Similarly, in the midbrain (Fig. 2C), 7-NI co-treatment in AED-treated rats decreased seizure-induced increase in LPO levels. 7-NI treatment increased the effect of diazepam and phenobarbitone by up to 35% and 34.4%, respectively.

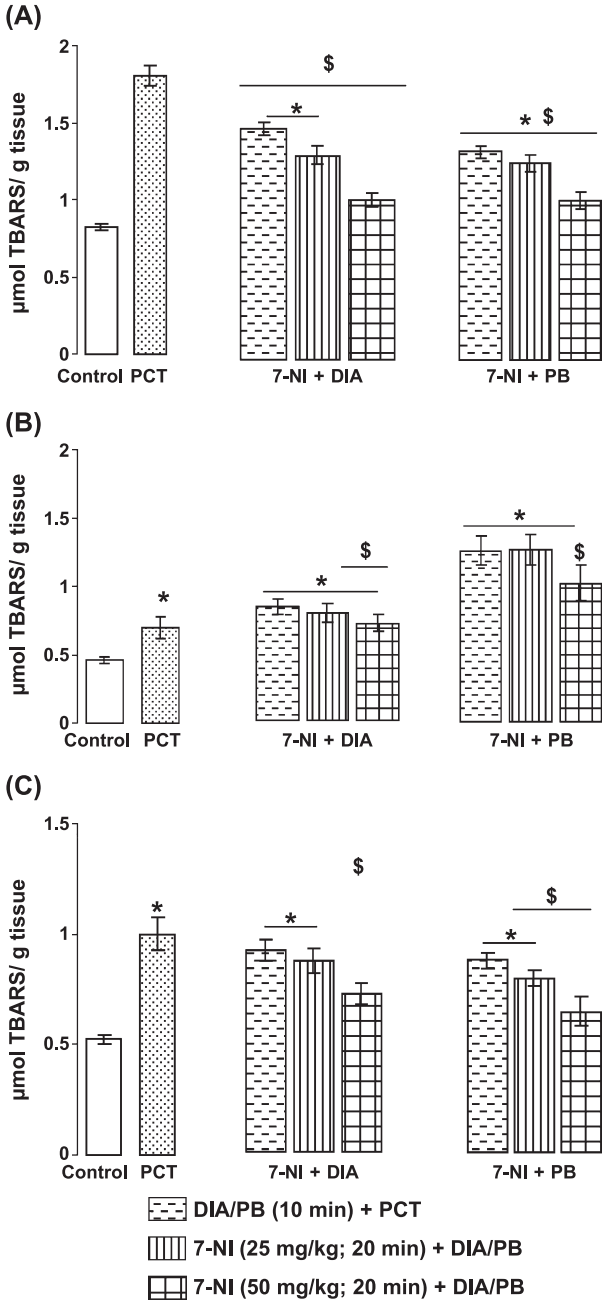

Fig. 2. Effect of co-treatment with 7-NI and AEDs on seizure-induced changes in the levels of TBARS in the (A) frontal cortex (B) hippocampus and (C) midbrain. Data represents mean±S.E.M. of 6 animals. *$P<0.05$ compared to control; \$$P<0.05$ compared to PCT-treated rats (ANOVA followed by Tukey's multiple comparison test).

3.3. Seizure-induced changes in the activity of antioxidant enzymes in rat brain regions: effect of 7-NI and AEDs

PCT-induced seizures differentially affected the activity of antioxidant enzymes in the rat brain regions. The effect of PCT, 7-NI and AEDs on the activity of SOD and GPx in rat brain regions are shown in Figs. 3 and 4, respectively. In the frontal cortex, there was a significant ($P<0.01$) increase in the activity of SOD (Fig. 3A), though the activity of GPx remained constant (data not shown). On the other hand, in the hippocampus, there was a significant ($P<0.001$; 53.8%) increase in the activity of SOD (Fig. 3B), together with a significant ($P<0.001$; 54.5%) decrease in the activity of GPx (Fig. 4A). In the midbrain, whereas, the activity of SOD remained constant (data not shown), the activity of GPx was significantly ($P<0.01$; 27.7%) decreased (Fig. 4B).

Treatment with 7-NI (50 mg/kg; 60 min) completely prevented PCT-induced increase in SOD activity in the frontal cortex and hippocampus. Also, 60 min of treatment with 7-NI at 25 mg/kg dose itself completely prevented

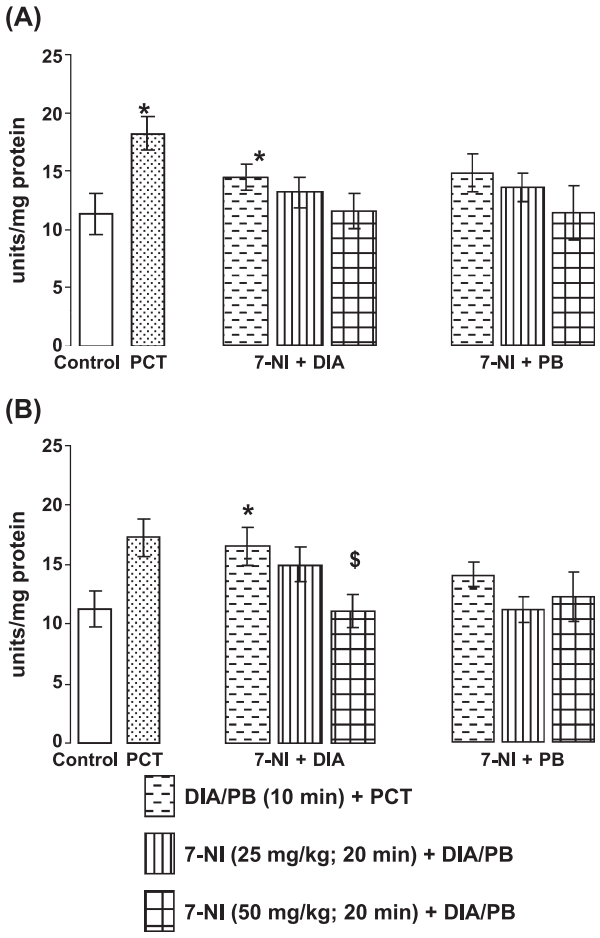

Fig. 3. Effect of co-treatment with 7-NI and AEDs on seizure-induced changes in activity of superoxide dismutase in rat (A) frontal cortex and (B) hippocampus. Data represents mean±S.E.M. of 6 animals. *P<0.05 compared to control; \$$P$<0.05 compared to PCT-treated rats (ANOVA followed by Tukey's multiple comparison test).

seizure-induced depletion in GPx activity in the hippocampus. On the other hand, in midbrain, the efficiency of 7-NI treatment in inhibiting seizure-induced GPx depletion increased with increasing doses and time points. Similarly, treatment with the AEDs could also significantly (P<0.05) decrease seizure-induced changes in the activity of antioxidant enzymes. Co-treatment with 7-NI enhanced the effects of AEDs and completely prevented seizure-induced changes in activity of antioxidant enzymes in all brain regions studied. In the frontal cortex, co-treatment with the smaller dose and treatment time of 7-NI (25 mg/kg; 20 min) significantly increased the effect of diazepam and phenobarbitone and completely inhibited seizure-induced increase in SOD activity (Fig. 3A). In the hippocampus (Fig. 3B), there was an increase in the effect of diazepam on SOD activity by co-treatment with 7-NI, the effect being more prominent with the higher dose (50 mg/kg). In contrast, in animals treated with phenobarbitone, the seizure-induced increase in SOD activity was completely prevented by the lower dose (25 mg/kg) of 7-NI itself (Fig. 3B).

7-NI co-treatment, with increasing dose and time, enhanced of the effect of diazepam in inhibiting seizure-induced decrease in GPx activity by a maximum of 80.2%. The effect of phenobarbitone on GPx activity was also increased by 7-NI by up to 71.5% to bring the levels to control values (Fig. 4). The effect of AEDs on seizure-induced decrease in GPx activity in the midbrain was significantly (P<0.01) increased by co-treatment with 7-NI (Fig. 4B). Increasing dose and treatment times with 7-NI progressively increased the effect of diazepam in inhibiting seizure-induced decrease in GPx activity. 7-NI treatment thus maximally enhanced the action of diazepam in reverting GPx activity to near normal values by up to 34%. On the other hand, similar to the effect observed in frontal cortex, co-treatment with the smaller dose and pretreatment time of 7-NI (25 mg/kg; 20 min) significantly enhanced (P<0.05) the action of phenobarbitone and completely prevented seizure-induced decrease in GPx activity (Fig. 4).

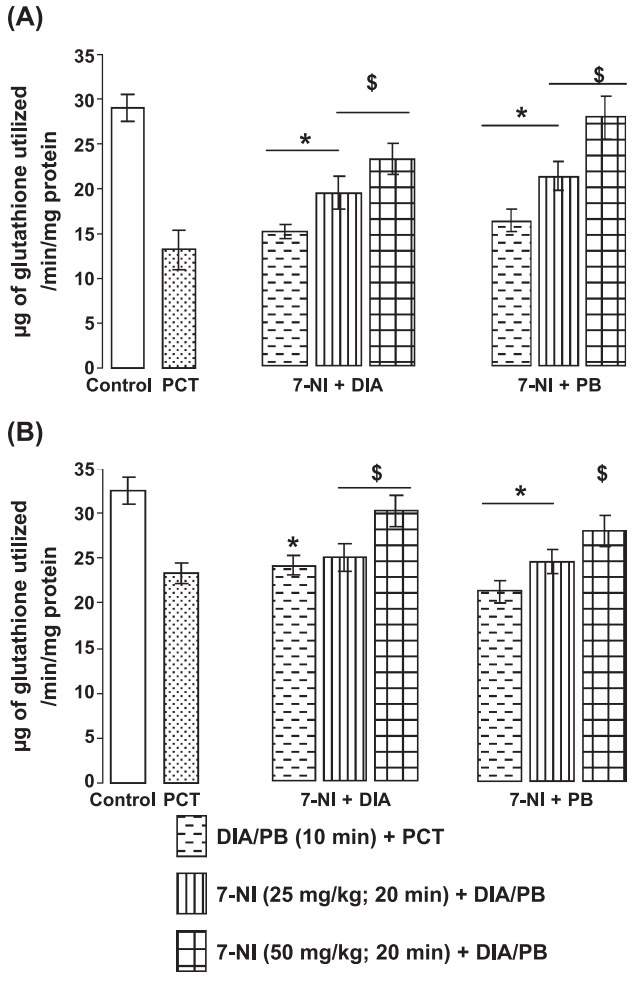

Fig. 4. Effect of co-treatment with 7-NI and AEDs on seizure-induced changes in activity of glutathione peroxidase in rat (A) hippocampus and (B) midbrain. Data represents mean±S.E.M. of 6 animals. *P<0.05 compared to control; \$$P$<0.05 compared to PCT-treated rats (ANOVA followed by Tukey's multiple comparison test).

4. Discussion

Picrotoxin is an inhibitory antagonist convulsant, frequently employed to produce generalized seizures when administered systemically. It acts at the *t*-butylcyclophosphorotionate/picrotoxin binding site on the $GABA_A$ receptor complex (Ito et al., 1989). PCT produces seizures by its interaction with the chloride ionophore (Fisher, 1989), thus blocking GABA-activated Cl^- currents, leading to disinhibition of excitatory processes and facilitation of the propagation of neuronal membrane depolarization (Maciejak et al., 2002). The $GABA_A$ receptors are highly sensitive to oxidative stress. Sah et al. (2002) have reported that free radicals could compromise with $GABA_A$-mediated neuronal inhibition leading to neuronal damage.

In an earlier study, we reported increased nNOS activity during PCT-induced seizures as early as 12 min and provided evidence for participation of nNOS inhibitory mechanism in the action of AEDs (Rajasekaran et al., 2003). The present study examined the role of the NO pathway in oxidative stress following PCT-induced seizures. The same experimental paradigm (including, the dose and treatment times of the drugs; and time of sacrifice) has been used in the current study to document the changes in LPO levels and action of antioxidant enzymes so as to compare the results of the this study with seizure data obtained in the previous report.

Biochemical estimates in the present study reveal regional differences. While the methodology adopted does not permit a speculation on the nature of seizure and consequent degree of injury with respect to the individual brain regions examined, these differences maybe attributed to at least two other factors. First, it may suggest a distinct pattern of neuronal involvement in PCT-induced seizures. Indeed, in the PCT model of seizures, it has been reported that the frontal cortex leads other brain structures in generalized spike and wave spindles associated with myoclonic jerks and seizure spikes that precede generalized convulsive seizures (Medvedev et al., 1996). Thus, the cortex appears to be the site of primary initiation, with the hippocampus and midbrain acting to generalize the seizures. Secondly, the regional differences observed in the study may also reflect variations in inherent mechanisms with regard to either free radical scavenging or increased potential for repair.

The results of the present study show that PCT-induced seizures increased the levels of NO in various brain regions, consistent with our earlier report that activation of nNOS triggers seizures induced by PCT (Rajasekaran et al., 2003). NO stimulates soluble guanylate cyclase, which raises the intracellular concentrations of cyclic guanosine monophosphate (cGMP). Since increased cGMP levels in brain during PCT-induced seizures have been reported (Ferrendelli et al., 1980), it is possible that the increases in cGMP content may involve prior receptor activation of nNOS with subsequent activation of guanylate cyclase by NO. A role for increased iNOS during the acute phase of seizures has also been reported in other experimental models of seizures (Murashima et al., 2002; Yang et al., 1999). Hence, it is likely that increased activity of iNOS may have also contributed to the elevated NO levels observed in this study. However, since the samples were processed in a way so as to exclude the membrane bound eNOS, and because iNOS occurs both in cytosolic and particulate fractions (Forstermann et al., 1991), we believe that there may have been only a partial contribution of iNOS-mediated NO release to the data obtained in the present study. Our observations are consistent with the reports of other investigators. Yasuda et al. (2001) reported that administration of the convulsant kainate resulted in increase of NO levels to 213% of basal level in the hippocampus and that the elevation was followed by epileptic discharges. We had previously reported that the doses of 7-NI employed in the present study possess anticonvulsant action per se and also potentiated the action of conventional AEDs by down-regulation of nNOS pathway (Rajasekaran et al., 2003). Earlier, Murashima et al. (2002) have reported that in the mutant EL mice, the content of nNOS (to a larger extent) and iNOS (to a lesser extent) were significantly increased during epileptogenesis, whereas eNOS content was completely diminished. The mutant EL mouse is a model of secondarily generalized seizures in which the parietal cortex has been reported to play a role in the initiation of seizures, with the hippocampus being responsible for generalization (Ishida et al., 1987). Together, these observations suggest a proconvulsant role for neuronally derived/inducible NO in seizures.

Both 7-NI and diazepam, administered either alone or together, were found to significantly reduce PCT-induced increase in NO levels in the brain regions. The results are in agreement with the observation of Mulsch et al. (1994) who reported that both diazepam as well as 7-NI could effectively inhibit KA-induced NO formation. While the effect produced by 7-NI can be attributed to its ability to inhibit nNOS activity directly, the mechanism by which AEDs decrease NO formation is still unclear. However, the apparent ability of diazepam to inhibit NO formation has been previously attributed, at least in part, to its structural similarity of the benzodiazepine ring nucleus with L-arginine (Fernandez-Cancio et al., 2001) that leads to a competitive inhibition of the NOS enzyme. Similarly, our observation of decrease in NO levels by phenobarbitone are also consistent with the report of Bashkatova et al. (1999) who reported a similar suppression of seizure-evoked NO generation in the rat cerebral cortex by pretreatment with phenobarbitone. These results point to the participation of NO inhibitory mechanisms in the action of AEDs. In the present study, the increase in LPO levels in brain regions 12 min following the injection of PCT corresponds to increases in regional NO levels. This observation suggests that NOS-mediated elevation of NO production could be potentially involved in the enhance-

ment of LPO during the development of seizures induced by PCT.

Seizure-induced oxidative injury could be the outcome of a multifactorial process that may primarily involve the over activation of the glutamate receptor (Coyle and Puttfarcken, 1993). Glutamate contributes to synaptic transmission when synaptic inhibition is depressed by $GABA_A$ antagonists (Herron et al., 1985); and also stimulates NO production by activation of the NMDA receptors which participate in the development and expression of epileptic seizures. The depolarization of neurons during epileptic activity promotes Ca^{2+} influx through voltage-activated channels and NMDA-gated ion channels, raising the intracellular Ca^{2+} (Uematsu et al., 1990). Such elevations in intracellular Ca^{2+} result in the depolarization of mitochondrial membranes and lead to an increase in intra-mitochondrial Ca^{2+} concentration (Duchen, 1992). As a consequence, respiration is increased and the concentration of reactive oxygen species elevated (Dugan et al., 1995). This in turn results in irreversible mitochondrial dysfunction in the neurons, possibly by initiation of peroxidation of lipids necessary for complex-IV activity by the free radicals (Bolanos et al., 1997) leading to a decrease in ATP production (Bolanos et al., 1995). In this context, the increase in LPO observed in the present study during seizures may be attributed to the potential activation of excitotoxic cascade initiated by the accumulation of extracellular glutamate following PCT administration (Sierra-Parades et al., 2000; Forman et al., 1998).

The increases in LPO levels in the cortex and hippocampus (but not midbrain) following administration of PCT could be significantly reduced by pretreatment with 7-NI and the AEDs, either alone or in combination, suggesting that LPO may be mainly derived from NO-related oxidative stress in these brain regions. It is likely that LPO in the midbrain is mainly derived from mechanisms other than NO-related oxidative stress, including dopamine auto-oxidation (Jacobsson et al., 1999). The neuroprotective effects of 7-NI may primarily relate to the inhibition of nNOS activity. Chavko et al. (2003) have demonstrated that seizures induce a significant increase in the production of protein nitrotyrosine–which is considered a footprint of peroxynitrite ($ONOO^-$)–and that nNOS inhibition by 7-NI retards seizures accompanied by reduction of seizure-induced increase in nitrotyrosine levels. Taken together, the results of the present study provide evidence for the participation of the NO pathway in contributing to oxidative stress during seizures and that the reactions mediated by the $NO/ONOO^-$ pathway may underlie one of the potential mechanisms contributing to seizures-induced neurotoxicity. It has previously been reported that AEDs could decrease LPO changes following neuronal insult. Mori et al. (1998) proposed that the mechanism of the novel AED, zonisamide may in part involve its ability to protect neurons by scavenging free radicals, including NO. Similarly, Bashkatova et al. (1999) have reported that phenobarbitone could completely suppress LPO induced by seizures in the rat cerebral cortex. Likewise, it has also been reported that diazepam could dose-dependently prevent psychological stress induced increase in LPO in the rat brain (Matsumoto et al., 1999). The exact mechanisms, however, remain unclear.

In the present study, we observed that seizure-induced alterations in the activity of antioxidant enzymes were region-specific. In the brain, SOD and GPx are considered to be the relatively more important antioxidant enzymes (Reiter, 1998). Hence, the significance in the alterations antioxidant enzyme activity should be viewed in the light of the changes in both SOD and GPx activities in the respective brain regions. We observed that in the frontal cortex and hippocampus, the activity of SOD during seizures was significantly increased. At the same time, the activity of GPx was unaltered in the frontal cortex, but decreased in the hippocampus.

SOD activity was determined to assess the rate of superoxide inactivation because of its possible impact on the rate of $ONOO^-$ formation in reaction with NO, since NO reacts with superoxide at a rate that is three times faster than the rate of inactivation by SOD (Huie and Padmaja, 1993). The Mn-SOD and Cu,Zn-SOD are key isoforms that play a key role in the cellular defense against oxidative processes that occur with the generation of superoxide. Mn-SOD is located in the mitochondria and preferentially expressed in terminally differentiating cells, whereas the Cu,Zn-SOD is located in the cytoplasm. A role for the SOD isoenzymes in modulating seizures and antiepileptic action has been proposed by Murashima et al. (1998). The authors reported that epileptic mutant EL mouse brain had significantly reduced Cu,Zn-SOD activity compared to non-epileptic controls, and that the antiepileptic effects of xanthine oxidase inhibitor, allopurinol, were associated with an increase in Cu,Zn-SOD activity. Interestingly, in the mutant EL mouse, the development and establishment of epilepsy was accompanied by an increase in the levels of nNOS, as well as iNOS, combined with a deficiency of eNOS (Murashima et al., 2000, 2002). Since the sites of initiation (cortex) and generalization (hippocampus) were comparable between this model and the PCT model, the reports of Murashima et al. (1998, 2000, 2002) provided us the rationale for investigating the effect seizures on SOD activity in the context of modulation of NO levels by 7-NI and AEDs.

In the present study, the increase in SOD paralleled an increase in LPO levels. The administration of 7-NI and AEDs decreased NO levels and prevented seizure-induced increase in SOD activities in the frontal cortex and hippocampus. The results obtained by us of increased SOD activity following seizures are consistent with the report of Chavko et al. (2003) who observed a significant increase in the activity of SOD following hyperoxia-induced seizures in rats. Our results are also in agreement with the earlier reports using a kainic acid model of seizures

by McIntosh et al. (1998) and Bruce and Baudry (1995). Increased SOD activity maybe an early compensatory reaction in response to increased NO and LPO levels, since SOD is one of the major antioxidant systems in the brain (Gluck et al., 2000; Hirata and Cadet, 1997). The increased activity of SOD observed in the present study is suggestive of an increase in the transcription of oxidative stress-inducible forms of SOD. However, it is very unlikely that such activation could occur as early as 12 min. Indeed, Dalton et al. (1995) had shown that the oxidative stress induced in the rat brain by kainic acid-induced seizures was not accompanied by transcriptional increase in the activity of various antioxidant enzymes, including SOD and GPx, to time points as long as 120 h after seizures. Thus, while transcriptional changes were not documented in the present study, it appears unlikely that transcriptional activation may have preceded the increase in SOD activity. Rather, the increase in SOD activity could be due to post-translational effects and activation of the preformed SOD.

Interestingly, some in vitro studies have suggested that GPx alone offered greater protection from oxidative stress than SOD in the brain (Erakovic et al., 2000). Indeed, a decrease in GPx activity, but not SOD even in normoxia, leads to toxicity (Michiels et al., 1994). The greater efficacy of GPx as an antioxidant may be attributed to the fact that it is located both in the cytosol and in the mitochondrial matrix. In this context, the observation of increased SOD activity together with decreased GPx activity in the hippocampus during PCT-induced seizures suggests the possibility of a potential oxidative damage to the hippocampus. The ability of 7-NI to inhibit formation of H_2O_2 (Mayer et al., 1994) may account for its ability to prevent seizure-induced decrease in hippocampal GPx activity. In the midbrain, whereas the activity of SOD was unchanged during seizures, the activity of GPx was significantly decreased. Combined treatment with 7-NI and AEDs significantly prevented seizure-induced changes in GPx activity, suggesting that the glutathione system may be the major free radical scavenging system in this region.

Interestingly, whereas both diazepam and phenobarbitone reduced PCT-induced increase in SOD activity in the frontal cortex, neither AEDs could completely restore the SOD activity to control values in the hippocampus. Furthermore, neither AEDs could significantly affect seizure-induced decrease in GPx activity in the hippocampus. These observations suggest that the AEDs may not have a significant role in modulating the activities of antioxidant enzymes and that their ability to decrease LPO is realized more likely by their ability to prevent the formation of free radicals.

In conclusion, the present study provides evidence for the involvement of the NOS pathway in seizure-induced oxidative stress. The study demonstrates the ability of the AEDs to inhibit seizure-induced increase in LPO levels, the effect being potentiated by pretreatment with 7-NI. It is likely that the protection against oxidative stress by these drugs could be the net outcome of their effects on the several potential pathways that influence cell injury following seizures. The observation that pretreatment with 7-NI and the AEDs could prevent the oxidative stress by acting on the nNOS pathway suggests that blockade of nNOS pathway has antioxidant actions and could possibly prevent seizure-induced neurotoxicity. These results warrant the need for further studies to understand the precise mechanism underlying their protection against seizure-induced oxidative stress.

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