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Antioxidant effect of nimodipine in young rats after pilocarpine-induced seizures

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Abstract

Nimodipine (ND) is a centrally active calcium antagonist that blocks the voltage-dependent L-type channels. Its antiepileptic properties have been proved in various animal models, including pilocarpine-induced seizures in adult rats. In order to investigate protective effects of the ND (10 (ND10) and 30 mg/kg (ND30), i.p.), young male rats (21-day-old) received ND injections before pilocarpine administration (400 mg/kg, s.c., pilocarpine group (P400)). The pretreatment with ND10 and ND30 prolonged the latencies of seizures and death on this seizure model. ND pretreatment in two doses decreased the levels of lipid peroxidation when compared to pilocarpine group. The P400 administration increased the striatal catalase activity. However, the administration of ND, in dose of 30 mg/kg, 30 min before pilocarpine, preserved catalase activity in normal levels. On the other hand, no change was detected in the animals treated with the dose of 10 mg/kg. Our results confirm the neuroprotective effect of ND on the seizures in young rats, suggesting that this drug acts positively on lipid peroxidation. Our observations shows that nimodipine cannot induces these effects via blockade of Ca^{2+}-channel.

Keywords: Nimodipine; Pilocarpine; Striatum; Lipid peroxidation; Catalase

1. Introduction

Status epilepticus is a neurologic emergency which has an associated mortality rate of 10–12%. This condition is characterized by prolonged or repetitive epileptic discharges, resulting clinically in persistent alterations of normal brain function and cognitive state (Treiman, 1995). Strong evidences link status epilepticus in childhood with the later development of epilepsy (Aicardi and Chevrie, 1983; Sagar and Osbury, 1987). Previous studies have shown that chronic seizures follow pilocarpine-induced SE in rats only if the drug is administered after the 18th day of life (Priel et al., 1996). Pilocarpine-induced seizure models have demonstrated behavioural and electroencephalograpic characteristics similar to those in human temporal lobe epilepsy (Turski et al., 1983).

The oxidative stress has been associated with seizure-induced neuronal death (Frantseva et al., 2000; Walz et al., 2000). The membrane lipid peroxidation, which is due to an increase in free radicals or decrease in activities of antioxidant defense mechanisms has been suggested to be accidentally involved in some forms of epilepsy (Jesberger and Richardson, 1991). The brain is a preferential target for the peroxidative process because it has a high content of polyunsaturated fatty acids (Halliwell and Gutteridge, 1984). Organisms have systems that prevent hazardous effects of free radicals such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-pX) and reduced glutathione (GSH) (Michiels et al., 1994). Recent studies suggest that differences are reported in free radical scavenging enzyme levels during the convulsive process (Freitas et al., 2004a).

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An increase in intracellular calcium concentration plays an important role in the epileptiform activity (Heinemann and Lux, 1983; Berg et al., 1995). Drugs that block the influx of calcium ions into the cells by blockade of voltage- or receptor-operated calcium channels are named calcium antagonists. They show antiepileptic properties in several in vitro and in vivo models of epilepsy (Walden et al., 1985; Speckmann and Walden, 1989; Wurpel and Iyer, 1994). Dihydropyridine compounds like nimodipine (ND) inhibit the neuronal Ca^{2+} entry through voltage-sensitive L-type Ca^{2+} channels. ND has been shown to have neuroprotective effects in many seizure models (Marinho et al., 1997; Kriz et al., 2003; Mikati et al., 2004). It also has been reported to prevent the increase in the brain free fatty acid levels in rats with penicillin-induced seizures, suggesting that the protection offered by ND may be independent of antagonisms on voltage-gated Ca^{2+} channels (Zupan et al., 1999).

The aim of this study was to investigate the effects of acute treatment with ND on behavioural changes, striatal lipid peroxidation levels and catalase activity on pilocarpine-induced seizures in young rats (21 days old).

2. Materials and methods

2.1. Animals

Male Wistar rats (40–50 g; 21 days old) were used. Animals were housed in cages with free access to food and water. All animals were kept with standard light–dark cycle (lights on at 07:00 h). The experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* of the U.S. Department of Health and Human Services, Washington, DC, 1985.

2.2. Drugs

Pilocarpine hydrochloride was purchased from ICN (CA, USA).

Nimodipine (Oxygen® 4%; solution) was obtained from Biosintética (Ceará, Brasil).

2.3. Treatment

Experiments were conducted at 8:00 a.m. in an experimental room. In a set of experiments, the animals were treated with nimodipine 10 or 30 mg/kg, i.p. (ND10 and ND30, respectively) or 0.9% saline, i.p., and 30 min later, they received pilocarpine hydrochloride, 400 mg/kg, s.c. (pilocarpine group—P400). Other two groups received ND10, ND30 or 0.9% saline (control). Animals were closely observed for behavioural changes (appearance of peripheral cholinergic reactions, such as miosis, piloerection, chromodacryorrhea, diarrhea, masticatory and stereotyped movements), latency to the development of the first seizure, *status epilepticus* and latency of death, immediately after the injection of pilocarpine during 1 h. The survivors were killed by decapitation and their brains were dissected on ice to remove striatum for determination of lipid peroxidation level and catalase activity. The pilocarpine group (P400) was constituted by those rats that presented seizures, SE for a period longer than 30 min and that did not died during 1 h.

2.4. Measurements of lipid peroxidation

Immediately after decapitation of the animals, the striatum was dissected for the preparation of homogenates 10% (w/v). The formation of lipid peroxides during lipid peroxidation process was analysed by measuring the thiobarbituric-acid-reacting substances (TBARS) in cells, as previously described by Huong et al. (1998). Briefly, the samples were mixed with 50 mM potassium phosphate monobasic buffer pH 7.4 and catalytic system of formation of free radicals ($FeSO_4$ 0.01 mM and ascorbic acid 0.1 mM), and then incubated at 37 °C for 30 min. The reaction was stopped with 0.5 ml of trichloroacetic acid 10%, then the samples were centrifuged (3000 rpm/15 min), the supernatants were retrieved e mixed with 0.5 ml of thiobarbituric acid 0.8%, then heated in a boiling water bath for 15 min and after this period, immediately cold in bath of ice. Lipid peroxidation was determined by the absorbance at 532 nm and was expressed as μmol of malondialdehyde (MDA)/g wet tissue.

2.5. Evaluation of catalase activity

Immediately after decapitation of the animals, the striatum was dissected and ultrasonically homogenized in 1 ml of 0.05 M phosphate buffer, pH 7.0, the protein concentration was measured according to the method described by Lowry et al. (1951) and used for catalase activity determinations. Catalase activity was measured by the method that employ hydrogen peroxide to generate H_2O and O_2 (Maehly and Chance, 1954). The activity was measured by degree of this reaction. The standard assay substrate mixture contained 0.30 ml of hydrogen peroxide in 50 ml of 0.05 M phosphate buffer, pH 7.0. The sample aliquot (20 μl) was added to 980 μl of substrate mixture. After 1 min, initial absorbance was recorded and final absorbance was read after 6 min. The reaction was followed at 230 nm. A standard curve was established using purified catalase (Sigma, MO, USA) under identical conditions. All samples were diluted with 0.1 mmol/l phosphate buffer (pH 7.0) to provoke an inhibition 50% of diluent rate (i.e. the uninhibited reaction) and results expressed as μM/min/μg protein (Maehly and Chance, 1954; Chance and Maehly, 1955).

2.6. Statistics analyses

Differences in experimental groups were determined by analysis of variance (ANOVA) two-tailed. The Student–

Newman–Keuls test was used for multiple comparison of means of groups of data whose differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Behavioural alterations of pre-treated with nimodipine young rats after pilocarpine-induced seizures

All animals treated with P400 presented peripheral cholinergic signs (miosis, piloerection, chromodacriorrhea, diarrhea, masticatory), and stereotyped movements (continuous sniffing, paw licking and rearing) followed by motor limbic seizures in 100% (40/40) of the tested animals. The convulsive process persisted and built up to a *status epilepticus* in 100% (40/40) of these rats, leading to death of 27.5% (11/40) of the animals (Table 1). The animals pretreated with ND10 and pilocarpine group (P400) developed cholinergic reactions, 71.4% (20/28) had seizures, 57.1% (16/28) built up to *status epilepticus* and 21.4% (6/28) died (Table 1). Peripheral cholinergic signs and stereotyped movements remained unaltered in group treated with ND30 and P400, 55.2% (21/38) had seizures, 44.7% (17/38) built up to *status epilepticus* and 21.0% (8/38) died (Table 1). ND administration, 30 min before P400, increased the latency to the onset of the first seizure (P400=7.16±0.35; ND10=12.98±1.02; ND30=25.26±2.10) and latency of death (P400=19.93±1.12; ND10=31.35±3.05; ND30=34.83±2.54) at two tested concentrations when compared to P400. No animals that received injections of isotonic saline (control) or ND (10 or 30) alone showed seizure activity.

3.2. Lipid peroxidation levels in striatum of young rats pretreated with nimodipine after pilocarpine-induced seizures

The treatment of animals with P400 (9.96±0.22) resulted in elevated levels of MDA when compared to control group (8.06±0.19). However when the groups were administered with the association of nimodipine and pilocarpine, the pretreatment with ND 10 (9.15±0.28) and 30 mg/kg (7.10±0.35) decreased the levels of lipid peroxidation when compared to P400 group alone (9.96±0.22). The animals treated only with ND 10 (7.23±0.14) or 30 mg/kg (7.01±0.34) did not show alteration on MDA levels when compared control group (8.06±0.19) (Fig. 1a and b). The results above were expressed as μmol of malondialdehyde (MDA)/g wet tissue.

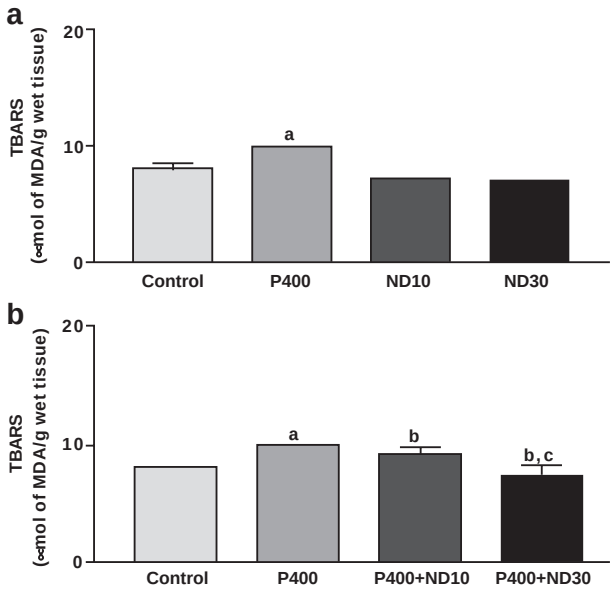

Fig. 1. (a) Acute effects of nimodipine on TBARS content in striatum of young rats after pilocarpine-induced seizures. Each bar represents the mean±S.E.M., $n=6-7$ rats/group. $^{a}p<0.05$ as compared to control group, by ANOVA followed by Student–Newman–Keuls. *Abbreviations:* **TBARS**—thiobarbituric-acid-reacting substances; **P400**—pilocarpine group; **ND**—nimodipine group. (b) Acute effects of nimodipine on TBARS content in striatum of young rats after pilocarpine-induced seizures. Each bar represents the mean±S.E.M., $n=6-7$ rats/group. $^{a,b,c}p<0.05$ as compared to control, P400, P400+ND10 groups, respectively, by ANOVA followed by Student–Newman–Keuls. *Abbreviations:* **TBARS**—thiobarbituric-acid-reacting substances; **P400**—pilocarpine group; **ND**—nimodipine group.

3.3. Determination of catalase activity in striatum of young rats pre-treated with nimodipine after pilocarpine-induced seizures

Results of CAT activity determination in striatum in 21-day-old rats are presented in Fig. 2a and b. The treatment with single dose of pilocarpine (71.66±6.96) produced increase in the catalase activity in comparison with the control group (37.24±3.40). The CAT activity, quantified in the animals treated only with ND 10 (39.74±3.94) or 30 mg/kg (39.64±3.04), showed similar value compared to that found for the control group (37.24±3.40). The group pretreated with ND 30 mg/kg before P400 (48.43±5.04)

Table 1
Behavioural changes in 21-day-old rats treated with pilocarpine (400 mg/kg, s.c.) and pre-treated with nimodipine (ND)

ND (mg/kg)	*n*	PCS (%)	Seizures (%)	Seizure's latency (min)	SE (%)	Death (%)	Death's latency (min)
–	40	100	100	7.16±0.35 ($n=40$)	100	27.5	19.93±1.12 ($n=11$)
10	28	100	71.4	12.98±1.02[a] ($n=20$)	57.1	21.4	31.35±3.05[a] ($n=6$)
30	38	100	55.2	25.26±2.10[a,b] ($n=21$)	44.7	21.0	34.83±2.54[a] ($n=8$)

Animals were observed during 1 h after P400 injection. Saline 0.9% (–) or different concentrations of ND, i.p., were administered 30 min before P400.
Abbreviations: **PCS**—peripheral cholinergic signs; **Seizures**—percentage of animals showing seizures; **Death**—percentage of death; **SE**—*status epilepticus*.
$^{a,b}p<0.001$ compared to control and ND10 groups, respectively, by ANOVA followed by Student–Newman–Keuls.

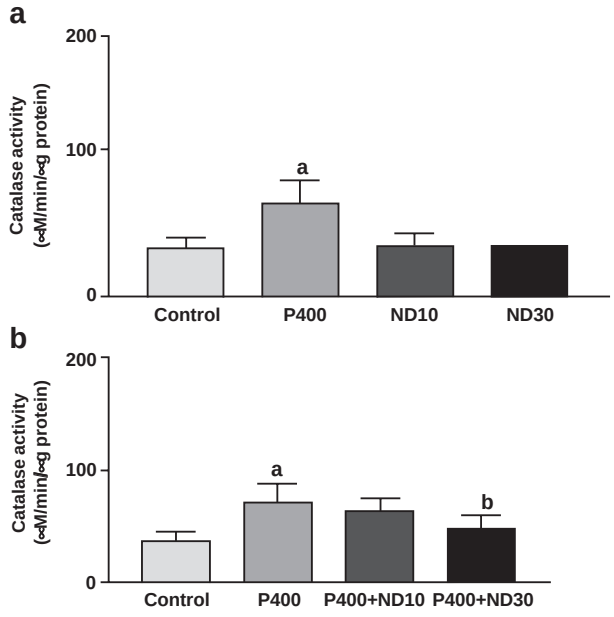

Fig. 2. (a) Acute effect of nimodipine on catalase activity in striatum of young rats after pilocarpine-induced seizures. Each bar represents the mean ± S.E.M., $n = 6-7$ rats/group. $^{a}p < 0.05$ compared to control, by ANOVA followed by Student–Newman–Keuls. *Abbreviations:* **P400**—pilocarpine group; **ND**—nimodipine group. (b) Acute effect of nimodipine on catalase activity in striatum of young rats after pilocarpine-induced seizures. Each bar represents the mean ± S.E.M., $n = 6-7$ rats/group. $^{a}p < 0{,}05$ compared to control, by ANOVA followed by Student–Newman–Keuls. *Abbreviations:* **P400**—pilocarpine group; **ND**—nimodipine group.

showed a decrease in relation to the treated P400 group alone (71.66 ± 6.96). On the other hand, no change was detected in the animals treated with the dose of 10 mg/kg (63.71 ± 3.87).

4. Discussion

The results of many experimental studies have shown that calcium channel blockers are effective against several different types of seizures (Van Luijtelaar et al., 2000; Zupan et al., 1999). For example, dihydropyridine calcium channel blockers have anticonvulsivant properties in several experimental models (Meyer et al., 1987; Charkrabarti et al., 1998; Zapatar et al., 1998; Kaminski et al., 1991) and ND, in particular, compared to other dihydropyridine calcium channel blockers, exert more potent anticonvulsant effect (Kriz et al., 2003). Our results confirm the anticonvulsant activity of ND on pilocarpine-induced seizures, as revealed by increases in the latency to the onset of seizures and latency of death, and reduction on seizures appearance and *status epilepticus*, after P400 administration.

Although the exact pathophysiological mechanism still needs to be clarified, the whole process can be related to the elevated intracellular concentration of Ca^{2+}. A variety of biochemical processes, including the activation of membrane phospholipases, proteases and nucleases which cause degradation of membrane phospholipids, proteolysis of cytoskeletone proteins and protein phosphorylation (Costa, 1994; Dugan and Choi, 1994) are triggered during seizures. In particular, polyphosphoinositides play an important role. Marked alterations in membrane phospholipid metabolism result in the liberation of free fatty acids (FFA), particularly free arachidonic acid, diacylglycerols, eicosanoids, lipid peroxides and free radicals (Costa, 1994). These lipid metabolites along with abnormal ion homeostasis and lack of energy generation may contribute to cell injury and death (Pellegrini-Giampietro et al., 1988; Shimizu and Wolfw, 1990; Simmet and Peskar, 1990). Erakovic et al. (2002) showed that the nimodipine in different doses (1, 3 and 10 mg/kg) reduce FFA level, but it did not influence glutathione peroxidase activity in cortex, cerebellum and hippocampus after lithium and pilocarpine-induced status epilepticus.

The pilocarpine, a cholinergic agonist, is widely used in studies of epilepsy as model of experimentally induced limbic seizures (Cavalheiro, 1995; Costa-Lotufo et al., 2002; Freitas et al., 2003). Freitas et al. (2004b) showed that lipid peroxidation levels are increased during the acute period of P400-induced seizures in adults rats. Our results also showed that P400 administration produced an increased lipid peroxidation in striatum of the young animals treated with P400, and therefore, demonstrated and confirmed the possible involvement of free radical oxygen injury in the P400 induced brain injury. The increase in free radical levels may be responsible for neuropathology induced by SE (Rong et al., 1999). There is, however, an endogenous antioxidant enzyme system against ROS-induced neuronal damage involving the cooperative action of SOD, CAT and GSH-Px. The SE also alter the free radicals scavenging enzymes activities, such SOD and CAT, indicating a cellular response to increased free radicals (Erakovic et al., 2000; Naffah-Mazzacoratti et al., 2001; Freitas et al., 2004a). SOD metabolizes O_2^- to H_2O_2, which is then detoxified by CAT and GSH-Px (Ferreira and Matsubara, 1997). Furthermore, we noted an increase in activity of the endogenous antioxidant catalase in striatum of young rats, 1 h after P400-induced seizures, confirming, thus, the defensive role of this molecule. Indeed, ND treatment decreased striatal MDA and preserved catalase activity in the normal levels, thereby suggesting that this drug acts positively on lipid peroxidation.

The mechanisms underlying the neuroprotective effect of ND are not fully understood, however our results are in agreement with protective effect of ND reported in previous studies in penicillin experimental model of seizure in which nimodipine was able to prevent an accumulation of the brain FFA in rats (Zupan et al., 1999), as well as Marinho et al. (1997) in the P400 model, showed that nimodipine did only not protect animals against status epilepticus, but was also able to decrease the cerebral damage significantly. In this way, our findings are in agreement with a growing line of evidence showing that nimodipine may not exclusively

modulate L-type of voltage-gated Ca^{2+} channel (Van Luijtelaar et al., 1995). It has been also shown that nimodipine blocks the efflux, rather than influx, of calcium from the synaptosomes, and therefore, it interferes with the mechanisms of neurotransmitter release and/or uptake from neuronal terminals (Woodward et al., 1988; Azmitia et al., 1993). The nimodipine neuroprotect effect in brain, can be responsible for influence the phenomena of excitotoxicity that has been related to an over production of free radicals by the tissue during pilocarpine-induced seizures and SE (Erakovic et al., 2000). In summary, the results of our present study, therefore, suggest that ND displays antioxidant activity that might explain, at least in part, the drug ability to behave as a possible neuroprotective and anticonvulsant agent.

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