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Crysophanol Effects on Lipid Peroxidation Levels and Catalase Activity in Mice Hippocampus after Pilocarpine-induced Seizures

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Abstract

Reactive oxygen species have been implicated in seizure-induced neurodegeneration, and there is a correlation between free radical level and scavenger enzymatic activity in the epilepsy. It has been suggested that pilocarpine-induced seizures is mediated by an increase in oxidative stress. Current research has found that antioxidant may provide, in a certain degree, neuroprotection against the neurotoxicity of seizures at the cellular level. Crysophanol has numerous nonenzymatic actions and is a powerful liposoluble antioxidant. The objective of present study was to evaluate the neuroprotective effects of crysophanol (CRY) in mice, against oxidative stress caused by pilocarpine-induced seizures, 30 min prior to behavioral observation, Swiss mice were treated with, 0.9% saline (i.p., control group), CRY (0.5 mg/kg, i.p., CRY 0.5 group), CRY (1.0 mg/kg, i.p., CRY 1.0 group), pilocarpine (400 mg/kg, i.p., P400 group), or the combination of CRY (0.5 or 1.0 mg/kg, i.p.) and pilocarpine (400 mg/kg, i.p.). After the treatments all groups were observed for 24 h. The enzymatic activities and lipid per oxidation concentrations were measured using spectrophotometric methods and these data were assayed. In P400 group mice there was a significant increase in lipid peroxidation levels and catalase activity. In the CRY and pilocarpine co-administered mice, antioxidant treatment significantly reduced the lipid peroxidation level, as well as increased the catalase activities in mice hippocampus after seizures. Our findings strongly support the hypothesis that oxidative stress occurs in hippocampus during pilocarpine-induced seizures, indicate that brain damage induced by the oxidative process plays a crucial role in seizures pathogenic consequences, and imply that strong protective effect could be achieved using crysophanol.

Keywords: Crysophanol; Hippocampus; Catalase; Status epilepticus; Seizures; Pilocarpine.

Introduction

Pilocarpine-induced status epilepticus (SE) produces many alterations in central nervous system neurotransmission. Previous work from our laboratory has demonstrated that intraperitoneal administration of pilocarpine 400 mg/kg significantly decreases not only muscarinic M_1, M_2 and Gabaergic receptors densities, but also decrease acetylcholinesterase (AChE) and increases superoxide dismutase and catalase enzymatic activities in the rat striatum, frontal cortex and hippocampus [1,2]. Recently, it was observed that the acute treatment with 400 mg/kg pilocarpine induces long-lasting alterations in serotonergic and glutamatergic receptors in rat frontal cortex, hippocampus and striatum [3].

Pilocarpine-induced seizure models have demonstrated behavioural and electroencephalograpic characteristics similar to those in human temporal lobe epilepsy [4]. 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 [5]. Strong evidences link status epilepticus in childhood with the later development of epilepsy [6].

The oxidative stress has been associated with seizure-induced neuronal death [7]. 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 [8]. The brain is a preferential target for the peroxidative process because it has a high content of polyunsaturated fatty acids [9]. 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) [10]. Recent studies suggest that differences are reported in free radical scavenging enzyme levels during the convulsive process [11,12].

Exogenous antioxidant like crysophanol (CRY) can inhibits the neuronal damage produced by lipid peroxidation in brain of the mice. Crysophanol has been shown to have antimicrobials effects in negative and positive gram bacterium (data not shown). It also has been reported to prevent the increase in the brain free fatty acid levels in mice with brain seizures, suggesting that the protection offered by crysophanol may be dependent of interactions with receptor different, but this mechanism remained understand.

Crysophanol can be believed to render protection against neurotoxicity, to a significant extent, role a free radical scavenging mechanism in the brain. It has been shown that exogenous antioxidants others significantly inhibits the hipocampal formation of MPP+, 30 min following systemic pilocarpine injection. Besides, neuroprotection by crysophanol has been reported in a recent work [13], showing that the compound presents a proliferative effect on the hippocampus. The aim of present study was to examine the effects of crysophanol on lipid peroxidation level and catalase activities in mice hippocampus prior to pilocarpine-induced seizures.

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Materials and Methods

Animals and experimental protocol

Male Swiss mice (25-30 g; 2-month-old), were obtained from Central Animal House of the Federal University of Piaui, Piaui, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant temperature (22 ± 1°C) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the official governmental guidelines in compliance with the Society Policy and was submitted by the Ethics Committee of the Federal University of Piaui, Brazil. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All doses are expressed in milligrams per kilogram and were administered in a volume of 10 ml/kg injected intraperitoneally (i.p.).

Drugs, seizures and status epilepticus

The following substances were used: pilocarpine hydrochloride (Sigma, Chemical USA) and crysophanol was isolated from *Senna reticulata*. All doses are expressed in milligrams per kilogram and were administered in a volume of 10 ml/kg injected intraperitoneally (i.p.).

Mice were pretreated with one of several doses of crysophanol (0.5 or 1.0 mg/kg, CRY), 30 minutes prior to intraperitoneal administration of pilocarpine 400 mg/kg, and in this 30-minute A total of 72 rats were treated with either 0.5 or 1.0 mg/kg crysophanol (i.p; CRY 0.5 or CRY 1.0) or 0.9% saline (i.p.). 30 min after the treatments 24 rats from each above group were randomized to pilocarpine hybrochloride administration (400 mg/kg, i.p., P400). Thus there are 6 groups of rats in this set of experiments: group 1, CRY 0.5 and P400 co-administration (n = 12); group 2, CRY 1.0 and P400 co-administration (n = 12); group 3, P400 plus saline treatment (n = 24); group 4, CRY 0.5 alone administration (n = 12); group 5, CRY 1.0 and P400 co-administration (n = 12); and group 6, saline treatment serves as control (n = 12). After the treatments, the animals were recorded in 30 X 30 cm chambers with: latency to first seizure (any one of the behavioral indices typically observed after pilocarpine administration: wild running, clonuses, tonus, clonic-tonic seizures), number of animals that died after pilocarpine administration. Previous work has shown that convulsions and deaths occurred within 1 and 24 h, respectively post pilocarpine injection, so we decided to record the phenotypes of the animals for 24 h after pilocarpine administration. At the end of observations, the survivors were killed by decapitation and their brains were dissected on ice to remove hippocampus for determinations of catalase activity. The pilocarpine administration rat group was constituted by those presented seizures, SE for over 30 min and non-phenotype survivors.

The animals that survived to pilocarpine treatment and control group were killed by decapitation 24 h after the treatment and their brains were dissected on ice to remove cerebral area (hippocampus) for determination of catalase activity. Detailed criteria to determine these periods after pilocarpine administration were reported previously by Cavalheiro et al. [14].

The drug dosages of pilocarpine (400 mg/kg) and crysophanol (0.5 and 1.0 mg/kg) [13] were determined by previous study in our lab [15,16] and the present study. The drug doses used in this present study are not equivalent to those used by humans because rats have different metabolic rates.

Determination of lipid peroxidation levels in hippocampus of the mice after seizures and status epilepticus

For lipid peroxidation levels determination, 10% (w/v) homogenates of the area of the brain investigated were prepared for all groups [17]. Lipid peroxidation levels in CRY 0.5 plus P400 group (n = 6), CRY 1.0 plus P400 group (n = 6); P400 group (n = 6), CRY 0.5 group (n = 6), CRY 1.0 group (n = 6) and control (n = 9) groups were analyzed by method previously described by Draper & Hadley [18]. The results above were expressed as nmol of malondialdehyde (MDA)/g wet tissue.

Catalase activity determinations in hippocampus of the mice after seizures and status epilepticus

Catalase activity was measured in the CRY 0.5 plus P400 group (n = 6), CRY 1.0 plus P400 group (n = 6); P400 group (n = 6), CRY 0.5 group (n = 6), CRY 1.0 group (n = 6) and control (n = 9) groups by the method that uses H_2O_2 to generate H_2O and O_2 [19]. Protein concentration was measured by the method of Lowry et al. [20]. The activity was measured by the degree of this reaction. The standard assay substrate mixture contained 0.30 ml H_2O_2 in 50 ml 0.05 M sodium phosphate buffer, pH 7.0. The sample aliquot (20 µl) was added to 980 µl of the substrate mixture. The initial and final absorbencies were recorded at 1 min and 6 min time-points respectively. The reaction was followed at 230 nm. A standard curve was established using purified catalase (Sigma, St Louis, MO, USA) under identical conditions. All samples were diluted with 0.1 mmol/L sodium phosphate buffer (pH 7.0), to provoke a 50% inhibition of the diluents rate (i.e. the uninhibited reaction). Results are expressed as U/mg protein [3,22].

Statistical analysis

Results of the latency to first seizure were compared using ANOVA and the Student–Newman–Keuls test as *post hoc* test, because these results show a parametric distribution. The number of animals that seized and the number that survived were calculated as percentages (% seizures and % survival, respectively) and compared with a nonparametric test (χ^2). In both situations statistical significance was reached at P less-than-or-equals, slant 0.05. The statistical analyses were performed with the software GraphPad Prism, Version 3.00 for Windows, GraphPad Software (San Diego, CA, USA).

Results

Pilocarpine induced the first seizure at 29.5 ± 1.46 min. 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 75% (9/12) of the tested animals ($p<0.0001$). The convulsive process persisted and built up to a status epilepticus in 75% (9/12) of these mice, leading to death of 75% of the animals (9/12) (Table 1). The animals pre-treated with CRY 0.5 and pilocarpine group (P400) developed cholinergic reactions, 100% (12/12) had seizures, 25% (06/12) built up to status epilepticus ($p<0.0001$) and 25% of the animal died (Table 1). CRY 0.5 administration, 30 min before P400, increased the latency to the onset of the first seizure in 163% (P400 = 29.5 ± 1.46; CRY 0.5 = 77.5 ± 1.72) ($p<0.05$), increased (25%) the survival rate ($p<0.0001$), when compared to the pilocarpine only group. The animals pre-treated with CRY 1.0 and pilocarpine group (P400) developed cholinergic reactions, 100% (12/12) had seizures, 25% (03/12) built up to status epilepticus ($p<0.0001$) and 25% of the animal died (Table

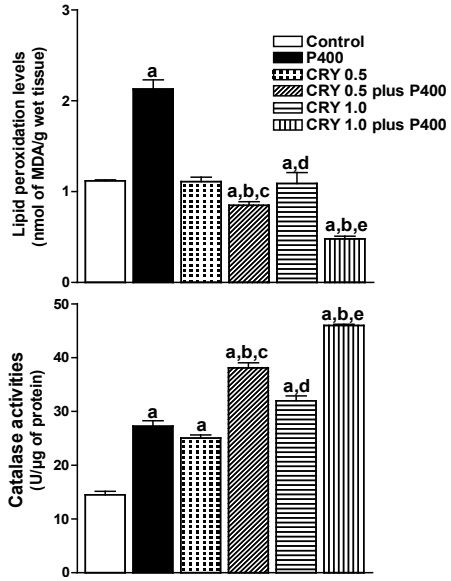
1). CRY 1.0 administration, 30 min before P400, increased the latency to the onset of the first seizure in 288% (P400 = 29.5 ± 1.46; CRY 1.0 = 114.5 ± 1.71) ($p<0.05$), and increased (50%) the survival rate ($p<0.0001$), when compared to the pilocarpine only group. No animals that received injections of isotonic saline (control), CRY 0.5 or CRY 1.0 alone showed seizure activity.

Effects of crysophanol on lipid peroxidation levels and catalase activities during seizures induced by pilocarpine are presented in Figure 1. Lipid peroxidation was markedly increased in P400 group in comparison with the corresponding values of the saline group. During acute phase of seizures induced by pilocarpine a significant increase (90%) in thiobarbituric-acid-reacting substances ($p<0.0001$) was observed. In addition, the pretreatment with crysophanol (0.5 mg/kg), 30 min before administration of pilocarpine also reduced lipid peroxidation level (40%, $p<0.0001$), when compared to the P400 group (Figure1). Por sua vez, the pretreatment with crysophanol (1.0 mg/kg), 30 min before administration of pilocarpine also reduced lipid peroxidation level (60%, $p<0.0001$), when compared to the P400 group (Figure 1). On the other hand, none of the control animals (saline or crysophanol) showed alterations in lipid peroxidation level (Figure 1). *Post-hoc* comparison of means indicated a decrease in lipid peroxidation levels of 24 and 58% in mice hippocampus in CRY 0.5 plus P400 [$T(12) = 6.044$; $p<0.0001$], and CRY 1.0 plus P400 [$T(12) = 3.9467$; $p<0.0001$], when compared with control group, respectively (Figure 1). An decrease in lipid peroxidation levels of of 44% in mice hippocampus in CRY 1.0 plus P400 [$T(12) = 9.044$; $p<0.0001$], when compared with CRY 0.5 plus P400 group. However, no was observed alteration lipid peroxidation concentration in CRY 1.0 group in comparison the CRY 0.5 group [$p>0.05$] (Figure 1).

Post-hoc comparison of means indicated an increase in the catalase activity of 85, 73, 162, 120 and 217% in the hippocampus in P400 [$T(13) = 2.3465$; $p<0.0001$], CRY 0.5 [$T(12) = 4.3465$; $p<0.0001$], CRY 0.5 plus P400 [$T(12) = 6.044$; $p<0.0001$], CRY 1.0 [$T(12) = 4.3465$; $p<0.0001$] and CRY 1.0 plus P400 [$T(12) = 3.9467$; $p<0.0001$] when compared with the control group, respectively (Figure 1). An increase in the catalase activities of 77, 35 and 132% in the hippocampus in CRY 0.5 plus P400 [$T(12) = 9.044$; $p<0.0001$], CRY 1.0 [$T(12) = 7.7044$; $p<0.0011$], and CRY 1.0 plus P400 [$T(12) = 5.9467$; $p<0.0001$] when compared with the P400 group, respectively. An increase of the 47% was observed in CRY 1.0 in relation the CRY 0.5 group [$T(12) = 5.9468$; $p<0.0001$]. In CRY 1.0 plus P400 was observed an increase of the 55% in relation CRY 0.5 plu P400 [$T(12) = 3.6457$; $p<0.0001$], respectively (Figure 1).

Groups	*Seizures (%)*	*Latency of first seizures (min)*	*Mortality rate (%)*	*Number of animals / group*
P400	75	29.5 ± 1.46	75	12
CRY 0.5 plus P400	50[a]	77.5 ± 1.72[c]	50[a]	12
CRY 0.5	00	00	00	12
CRY 1.0 plus P400	20[a,b]	114.5 ± 1.71[c,d]	20[a,b]	12
CRY 1.0	00	00	00	12

Table 1: Effect of pretreatment with crysophanol (CRY) on pilocarpine-induced seizures and lethality in adult rats. Male mice (25-30 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, intraperitoneal, i.p., n = 6, P400 group); CRY 0.5 or CRY 1.0 group with crysophanol (0.5 mg/kg, i.p., n = 6, CRY 0.5 group); CRY 1.0 group with crysophanol (1.0 mg/kg, i.p., n = 6, CRY 1.0 group) and the control animals with 0.9% saline (i.p., n = 9, Control). The CRY 0.5 plus pilocarpine group was treated with crysophanol (0.5 mg/kg, i.p.) for 30 min prior to pilocarpine injection (400 mg/kg, i.p., n = 6, CRY 0.5 plus P400) and CRY 1.0 plus pilocarpine group was treated with crysophanol (1.0 mg/kg, i.p.) for 30 min prior to pilocarpine injection (400 mg/kg, i.p., n = 6, CRY 1.0 plus P400). Results for latency to first seizure are expressed as means ± SEM of the number of experiments shown in the table. Result for % seizures and % survival are expressed as percentages of the number of animals from each experimental group. [a]$p<0.05$ as compared with pilocarpine group (χ^2 test); [b]$p<0.05$ as compared with CRY 0.5 (χ^2 test); [c]$p<0.0001$ as compared with P400 group (ANOVA and *t*-Student-Newman-Keuls test); [d]$p<0.0001$ as compared with CRY 0.5 (ANOVA and *t*-Student-Newman-Keuls test).



[a]$p<0.05$ as compared with control group (ANOVA and *t*–Student–Neuman–Keuls as *post hoc* test).
[b]$p<0.05$ as compared with P400 group (ANOVA and *t*–Student–Neuman–Keuls as *post hoc* test).
[c]$p<0.05$ as compared with CRY 0.5 plus P400 (ANOVA and *t*–Student–Neuman–Keuls as *post hoc* test).
[d]$p<0.05$ as compared with CRY 0.5 (ANOVA and *t*–Student–Neuman–Keuls as *post hoc* test).
[e]$p<0.05$ as compared with CRY 1.0 plus P400 (ANOVA and *t*–Student–Neuman–Keuls as *post hoc* test).

Figure 1: Effects of the crysophanol (0.5 e 1.0 mg/kg) on lipid peroxidation levels and catalase activities in hippocampus of adult mice after status epilepticus induced by pilocarpine. Male mice (25–30 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, intraperitoneal, i.p., n = 6, P400 group); CRY 0.5 or CRY 1.0 group with crysofanol (0.5 mg/kg, i.p., n = 6, CRY 0.5 group); CRY 1.0 group with crysofanol (1.0 mg/kg, i.p., n = 6, CRY 1.0 group) and the control animals with 0.9% saline (i.p., n = 9, Control). The CRY 0.5 plus pilocarpine group was treated with crysonafol (0.5 mg/kg, i.p.) for 30 min prior to pilocarpine injection (400 mg/kg, i.p., n = 6, CRY 0.5 plus P400) and CRY 1.0 plus pilocarpine group was treated with crysonafol (1.0 mg/kg, i.p.) for 30 min prior to pilocarpine injection (400 mg/kg, i.p., n = 6, CRY 1.0 plus P400). Results are expressed as means ± S.E.M. for the number of animals shown inside in parenthesis. Differences in experimental groups were determined by two-tailed analysis of variance (ANOVA) and *t*–Student–Neuman–Keuls as *post hoc* test.

Discussion

Epilepsy is one of the most common neurologic problems all over the world, being associated with paroxysmal discharge of cerebral neurons and is characterized by several symptoms including alterations of behaviors and consciousness [23]. The molecular observations of epilepsy include the temporal correlation between free radical generation and the development of seizures in some pathological conditions, and the protective efficacy of antioxidative treatments against some types of seizures. Previous studies indicated that crysophanol has anticonvulsant activity in epilepsy animal models induced by pilcoarpine [13]. In this study, we demonstrated a role of crysophanol against lipid peroxidation generated by pilocarpine-induced seizures.

In the present study we investigated the influence of crysophanol

on lipid peroxidation level and enzymatic activities of catalase in the mice hippocampus during pilocarpine-induced seizures. Generation of reactive oxygen species is currently viewed as one of the process through which epileptic activity exert their deleterious effects on brain [11,12]. These reactive oxygen species in the absence of an efficient defense mechanism cause peroxidation of membrane polyunsaturated fatty acids [23,24]. Brain is particularly susceptible to peroxidation due to simultaneous presence of high levels of polyunsaturated fatty acids and iron [25,26] which are the targets of free radical damage. We showed the lipid peroxidation was rising in hippocampus homogenate of mice after 24 h of acute phase of seizures. The increase of lipid peroxidation was reflected by the rise of thiobarbituric-acid-reacting substances level which may be related to its intermediate free radicals formed during seizures induced by pilocarpine.

Very recently, the administration of exogenous drugs antioxidants was demonstrated to cause a synergistic effect on catalase activity [21]. According to the authors, the positive effects of exogenous drugs antioxidants on catalase activity may be due, at least in part, to its antioxidant activity [19,22].

In the present work, we showed that mice submitted to seizures and status epilepticus presented a significant increase in the latency to first seizures and decrease in mortality rate, as compared to controls, indicating an anticonvulsive effect. Vitamin C and glutathione reduced significantly reversed the mortality rate observed after status epilepticus in adult rats [12,16].

It has been shown that vitamin C and glutathione reduced improves learning memory processes, in some cognitive tests in aged rats by scavenging of free radicals. The data suggest that vitamin C possesses potential cognitive enhancement abilities, probably due to an increase in the catalase activity [12,16].

Freitas et al. [19] showed that P400 caused a threefold increase in superoxide dismutase and catalase activities in rat striatum, hippocampus and frontal cortex. Similar results were seen with crysophanol, in mice after seizures and status epilepticus induced by pilocarpine.

In the present work, was observed an increase in the catalase activity after seizures and status epilepticus in mice treated with crysophanol in both doses. The catalase activity present high activity in P400 + CRY 1.0 due to the dose high of this compound, acting more intensively on hippocampal neurons. On the other hand, the catalase activities increases previously in CRY 1.0 group and seen in the CRY 0.5 group was completely increase as compared. In conclusion, our results indicate that the crysophanol treatment increase the latency the first seizures and status epilepticus, and reduce the mortality rate in adult mice. In mice submitted to seizures and status epilepticus induced by pilocapine was observed antioxidants effects of the crysophanol. CRY in both doses produced increases in catalase activity hippocampal, suggesting radical free production reduction in brain of epileptic mice. While these effects were also observed after seizures and status epilepticus in hippocampus of adult rats treated with vitamin C. In summary, our study provides convincing evidence for the involvement of the catalase enzymatic activity in the activation of cholinergic neurons in brain adult mice during the establishment of status epilepticus.

Conclusions and Future Perspectives

Herein, we clearly showed that crysophanol decreased the frequency of pilocarpine-induced seizures and increased the survival rate. In our knowledge, these effects of crysophanol on oxidative stress observed during acute phases of pilocarpine-induced seizures have not been reported before. Thus, these findings might have important implications for understanding the mechanism of epilepsy to promote new advances in the development of selective and targeted antiepileptic drugs. Crysophanol protected the hippocampus against neuronal damages regularly observed during seizures. Further investigations of crysophanol effects against necrosis, apoptosis and/or autophagy observed during the acute phase of this epilepsy model are in progress to confirm its neuroprotective effects.

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