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Pharmacology, Biochemistry and Behavior 78 (2004) 327–332

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Pilocarpine-induced status epilepticus in rats: lipid peroxidation level, nitrite formation, GABAergic and glutamatergic receptor alterations in the hippocampus, striatum and frontal cortex

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Received 6 December 2003; received in revised form 4 April 2004; accepted 8 April 2004
Available online 14 May 2004

Abstract

The aim of the study was to investigate the lipid peroxidation levels, nitrite formation, GABAergic and glutamatergic receptor densities in the hippocampus, frontal cortex and striatum of Wistar rats after seizures and status epilepticus (SE) induced by pilocarpine. The control group was treated with 0.9% saline and sacrificed 1 h after the treatment. One group of rats was administered with pilocarpine (400 mg/kg sc) and sacrificed 1 h after treatment. The result shows that pilocarpine administration and the resulting SE produced a significant increase of lipid peroxidation level in the hippocampus (46%), striatum (25%) and frontal cortex (21%). In nitrite formation, increases of 49%, 49% and 75% in hippocampus, striatum and frontal cortex, respectively, was observed. Pilocarpine treatment induced down-regulation of GABAergic receptors in the hippocampus (38%), striatum (15%) and frontal cortex (11%). However, with regard to glutamatergic receptor densities, increases in the hippocampus (11%), striatum (17%) and frontal cortex (14%) was observed during the observation period. These results show a direct evidence of lipid peroxidation and nitrite formation during seizure activity that could be responsible for the GABAergic and glutamatergic receptor concentration changes during the estabilishment of SE induced by pilocarpine.

Keywords: Seizures; Lipid peroxidation; Nitrite formation; GABAergic receptor; Glutamatergic receptor; Pilocarpine

1. Introduction

Epilepsy is the most common neurological disorder after stroke, with 0.5% prevalence (Browne and Holmes, 2001). The acute administration of a high dose of pilocarpine in rodents is an experimental model largely used to study the pathophysiology of seizures (Freitas et al., 2003b). This seizure model demonstrates the potent proconvulsive and damaging effect of pilocarpine on rat brain (Turski et al., 1983). The subcutaneous administration of pilocarpine produces a sequence of behavioural alterations (peripheral cholinergic signs, tremors, staring spells, facial automatisms and motor limbic seizures), which develop progressively, between 1 and 2 h, into a several hour limbic status epilepticus (SE; Cavalheiro et al., 1991; Freitas et al., 2003a).

The epilepsy model induced by a high dose of pilocarpine has revealed behavioural and electroencephalographic alterations that are similar to those in human temporal lobe epilepsy (Turski et al., 1983). In rats, this model is characterized by an acute phase, which is characterized by animals presenting long-lasting SE (1–2 days), by a seizure-free period (silent; 4–44 days, mean of 15 days) and by chronic phase, characterized by spontaneous recurrent seizures (SRS; Leite et al., 1990; Cavalheiro et al., 1991, 1994).

Neurochemical studies (neurotransmitter and receptor density) have been performed after the convulsive process (Marinho et al., 1998; Erakovic et al., 2000; Costa-Lotufo et al., 2002; Freitas et al., 2003b). Seizure activity with a wide range of local biochemical changes affects several neurotransmitters [adenosine, noradrenaline, dopamine, serotonine, glutamate, γ-aminobutyric acid (GABA); Cavalheiro et al., 1991], and ($M_1 + M_2$) muscarinic and (D_1- and D_2-like) dopaminergic receptor densities in the hippocampus (Marinho et al., 1998) and striatum (Freitas et al., 2003b) induced by pilocarpine.

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doi:10.1016/j.pbb.2004.04.004

The mechanism of pilocarpine-induced SE and the produced neuronal damage is unclear. Following the toxicity induced by an initial cholinergic phase, a distinct non-cholinergic phase occurs, in which excessive glutamate and aspartate release induces SE in this epilepsy model (McDonough and Shih, 1997; Solberg and Belkin, 1997).

It is currently hypothesized that some pathological processes (such as SE), which increase glutamate release, activate a higher number of glutamatergic receptors for a critical period of time, leading to neuronal necrosis by elevating $[Ca^{+2}]_i$ and activating potentially destructive Ca^{+2}-dependent enzymes (Fujikawa et al., 1994). The increase of these Ca^{+2}-dependent enzyme activity can induce an oxidative stress which has been implicated in a variety of acute and chronic neurologic conditions, including SE (Walz et al., 2000). Nevertheless, it is not well established if the reactive oxygen species (ROS) play a role in pilocarpine-induced seizures.

The increase in ROS levels can also be responsible for neuropathology induced by SE (Rong et al., 1999). The SE also activates the ROS scavenging enzymes, such as superoxide dismutase (SOD) and catalase, indicating a cellular response to increased ROS (Ferrer et al., 2000). SE induces ROS production-mediated protein oxidation as measured by tyrosine nitration (Rong et al., 1999), as well as lipid peroxidation as indicated by malondialdehyde (Bruce and Baudry, 1995). That being so, it is important to investigate lipid peroxidation levels and nitrite content during the acute phase of seizures induced by pilocarpine.

The role of cholinergic and dopaminergic receptors in the process of seizures is not clear either and several authors have suggested that the activation of cholinergic receptors is necessary in the initiation of the limbic seizures and that other systems (serotonergic, GABAergic and glutamatergic) may be related to seizures and SE (Turski et al., 1989; Costa-Lotufo et al., 2002; Freitas et al., 2003c).

It is possible that dopaminergic, serotonergic, GABAergic and glutamatergic receptors may also participate in the convulsive process, but when and how it happens, has to be determined. Considering that receptor density alterations and that the relation to other neurochemical changes involved in seizure mechanisms are still unclear, this work was performed to determine the lipid peroxidation level, nitrite content, GABAergic and glutamatergic receptor concentrations in different cerebral regions (hippocampus, frontal cortex and striatum) of adult rats during the acute period of seizures induced by pilocarpine.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–280 g; 2 months 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 0700 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 and chemicals

Pilocarpine hydrochloride was purchased from ICN (California, USA). Radioligands [^{3}H]-GABA (81 Ci/mmol) and [^{3}H]-glutamate (114 Ci/mmol) were provided by Amersham Pharmacia Biotech (New Jersey, USA). All other drugs were of analytical grade.

2.3. Experimental procedure

Control animals received 0.9% saline subcutaneously (control group; $n=35$) and in the other group, the animals were treated with a single dose of pilocarpine hydrochloride (400 mg/kg sc; $n=25$). Behavioural changes were observed during 1 h. The observed parameters were as follows: number of peripheral cholinergic signs, tremors, stereotyped movements, seizures, SE and mortality. The SE was defined as continuous seizures for a period longer than 30 min. SE was induced by the method of Turski et al. (1983). Mortality was recorded 1 h after pilocarpine-induced SE.

The pilocarpine group was constituted by those rats that presented seizures, SE and those that did not die during 1 h of observation after the treatment. The animals that survived to pilocarpine treatment and the control group were killed by decapitation 1 h after the treatment and their brains were dissected on ice to remove cerebral areas (hippocampus, frontal cortex and striatum) for determination of lipid peroxidation level, nitrite content, GABAergic and glutamatergic receptor binding assays.

2.4. Evaluation of lipid peroxidation

Immediately after decapitation of the two groups (pilocarpine and control groups), hippocampus, frontal cortex and striatum were dissected for the preparation of the homogenates 10% (w/v). The formation of lipid peroxides during lipid peroxidation process were analysed by measuring the thiobarbituric-acid-reacting substances (TBARS) in cells, as previously described by Draper and Hadley (1990). Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, then heated in a boiling water bath for 15 min, and butanol (2:1 v/v) was added to the solution. After centrifugation ($800 \times g$/5 min), the TBARS were determined by the absorbance at 535 nm.

2.5. Nitrite determination

Immediately after decapitation of the two groups (pilocarpine and control groups), hippocampus, frontal cortex

and striatum were dissected for the preparation of the homogenates 10% (w/v). After centrifugation (800 × *g*/10 min), the supernatant of homogenate was collected and the production of NO was determined based on Griess reaction (Green et al., 1981). Briefly, 100 μl of supernatant was incubated with 100 μl of the Griess reagent (1% sulfanilamide in 1% H_3PO_4/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/1% H_3PO_4/distilled water, 1:1:1:1) at room temperature for 10 min. The absorbance was measured at 550 nm via a microplate reader. Nitrite concentration was determined from a standard nitrite curve generated by using $NaNO_2$.

2.6. GABAergic and glutamatergic receptor density determinations

Immediately after decapitation of the two groups (pilocarpine and control groups), hippocampus, frontal cortex and striatum were dissected for the preparation of the homogenates 10% (w/v). Homogenates were prepared in 2 ml of 0.32 M sucrose, according to the method described by Vogel and Vogel (1997). Homogenates were centrifuged at 20,000 × *g* for 10 min at 4 °C and the pellets were suspended in distilled water, and centrifuged at 20,000 × *g* for 15 min at 4 °C. The resulting pellets were suspended in ice-cold 50 mM Tris–HCl buffer followed by centrifugation at 20,000 × *g* for 15 min at 4 °C, resuspended in Triton X-100 0.05%, and incubated for 15 min at 37 °C. The procedures performed with Triton X-100 in homogenates of both groups were carried out under the same conditions. After the incubation, the assay mixture was washed two times in 50 mM Tris–HCl buffer at 4 °C and centrifuged at 20,000 × *g* for 15 min.

The final pellet was then suspended in 0.3 ml of 50 mM Tris–HCl buffer. Binding was performed incubating 0.05 ml of the membrane preparation (0.3–0.5 mg protein) with 50 nM [^{3}H]-GABA (81 Ci/mmol, Amersham Pharmacia Biotech) in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of muscimol (100 μM). The incubation time was 30 min at 37 °C.

In the case of glutamatergic receptors, binding was performed incubating 0.05 ml of the membrane preparation (0.3–0.5 mg protein) with 50 nM of [^{3}H]-glutamic acid (49 Ci/mmol, Amersham Pharmacia Biotech) in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of glutamic acid (1 μM). The incubation time was 30 min at 37 °C.

After all incubation times, the samples were filtered through Whatman GF/B filters. Filters were washed three times with 4 ml of ice-cold saline, dried and added to 3 ml of a toluene-based scintillation cocktail. Radioactivity was measured with a Beckman scintillation counter model LS 6500 and results were expressed as fentomoles per milligram of protein (fmol/mg protein).

Protein was determined using bovine serum albumin as the standard (Lowry et al., 1951).

2.7. Statistics

Student–Newman–Keuls test was used for multiple comparison of means of two groups of data whose differences were considered statistically significant at $P < 0.05$. Differences in experimental groups were determined by analysis of variance (ANOVA), two-tailed.

3. Results

3.1. Behavioural alterations of adult rats after pilocarpine-induced SE

Immediately after pilocarpine administration, animals persistently had behavioural changes, including initial akinesia, ataxic lurching, peripheral cholinergic signs (miosis, piloerection, chromodacriorrhea, diarrhea and masticatory automatisms), stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes that persisted for 10–15 min), clonic movements of forelimbs, head bobbing and tremors. These behavioural changes progressed to motor limbic seizures as previously described by Turski et al. (1983). Limbic seizures persisted for 30–50 min evolving to SE in all rats. During the behavioural study, none of the animal died.

3.2. Lipid peroxidation levels in hippocampus, striatum and frontal cortex of adult rat after pilocarpine-induced SE

The lipid peroxidation level (TBARS formed) in the brain homogenates was markedly increased in this model when compared to control group. After pilocarpine-induced SE, significant increases of 46%, 25% and 21% was

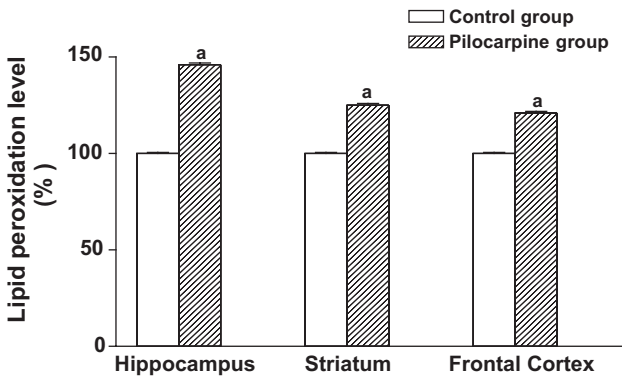

Fig. 1. Determination of the lipid peroxidation levels in hippocampus, striatum and frontal cortex of adult rat after pilocarpine-induced SE. Rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg sc; $n = 7$) and the control group with 0.9% saline ($n = 9$). Animals were submitted to a 1-h observation and afterwards were sacrificed. Each bar represents the mean ± S.E.M. of the number of animals shown in parentheses. (a) $P < 0.05$ as compared to control animals (Student–Newman–Keuls test) and the differences in experimental groups were determined by ANOVA.

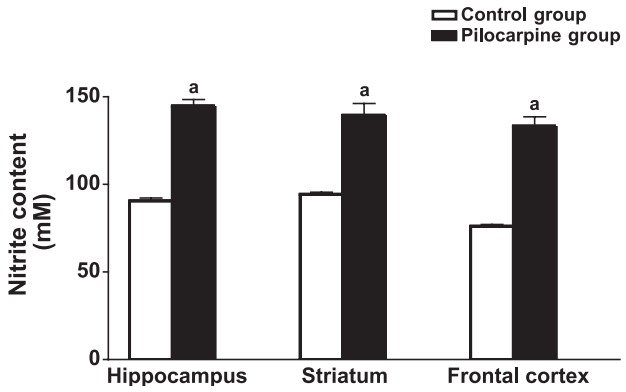

Fig. 2. Determination of the nitrite content in hippocampus, striatum and frontal cortex of adult rat after pilocarpine-induced SE. Rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg sc; $n=10$) and the control group with 0.9% saline ($n=10$). Animals were submitted to a 1-h observation and afterwards were sacrificed. Each bar represents the mean ± S.E.M. of the number of animals shown in parentheses. (a) $P<0.05$ as compared to control animals (Student–Newman–Keuls) and the differences in experimental groups were determined by ANOVA.

measured in the hippocampus [$t(14)=19.067$; $P<0.0001$], striatum [$t(10)=3.855$; $P<0.0032$] and frontal cortex [$t(11)=4.881$; $P<0.0005$] in lipid peroxidadion levels, respectively, when compared to control group (Fig. 1).

3.3. Nitrite levels in hippocampus, striatum and frontal cortex of adult rats after pilocarpine-induced SE

Pilocarpine-induced SE produced in nitrite content had significant increases of 49%, 49% and 75% in hippocampus [$t(18)=13.424$; $P<0.0001$], striatum [$t(18)=6.655$; $P<0.0001$] and frontal cortex [$t(18)=10.728$; $P<0.0001$], respectively, when compared to control group (Fig. 2).

3.4. GABAergic and glutamatergic receptor densities in hippocampus, striatum and frontal cortex of adult rats after pilocarpine-induced SE

The total number of [^{3}H]-GABA and [^{3}H]-glutamic acid binding sites was obtained from the hippocampus, striatum and frontal cortex membranes of 2-month-old animals after pilocarpine-induced SE (Tables 1 and 2), respectively.

Table 1
GABAergic receptor densities (B_{max}) in hippocampus, striatum and frontal cortex of adult rats after pilocarpine-induced SE

Group	B_{max} (fmol/mg protein)		
	Hippocampus	Striatum	Frontal cortex
Control	746.25 ± 10.92 (8)	747.75 ± 13.00 (8)	794.25 ± 1.32 (8)
Pilocarpine	458.50 ± 21.88 (4)[a]	637.50 ± 39.28 (4)[a]	707.75 ± 10.26 (4)[a]

Rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg sc) and the control group with 0.9% saline. Animals were submitted to a 1-h observation and afterwards were sacrificed. The values represent the mean ± S.E.M. of the number of animals shown in parentheses.

[a] $P<0.05$ as compared to control animals (Student–Newman–Keuls) and the differences in experimental groups were determined by ANOVA.

Table 2
Glutamatergic receptor densities (B_{max}) in hippocampus, striatum and frontal cortex of adult rats after pilocarpine-induced SE

Group	B_{max} (fmol/mg protein)		
	Hippocampus	Striatum	Frontal cortex
Control	974.38 ± 8.11 (8)	1003.35 ± 1.19 (8)	995.25 ± 8.92 (8)
Pilocarpine	1085.78 ± 28.16 (4)[a]	1173.75 ± 15.29 (4)[a]	1135.50 ± 12.04 (4)[a]

Rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg sc) and the control group with 0.9% saline. Animals were submitted to a 1-h observation and afterwards were sacrificed. The values represent the mean ± S.E.M. of the number of animals shown in parentheses.

[a] $P<0.05$ as compared to control animals (Student–Newman–Keuls) and the differences in experimental groups were determined by ANOVA.

A down-regulation in GABAergic receptors of 38%, 15% and 11% was observed after 1 h in the hippocampus [$t(10)=13.329$; $P<0.0001$], striatum [$t(10)=3.403$; $P<0.0067$] and frontal cortex [$t(10)=12.111$; $P<0.0001$] after pilocarpine-induced SE, respectively, when compared to control group (Table 1).

However, in glutamatergic receptor densities after pilocarpine-induced SE in adult rats, increases of 11%, 17% and 14% was verified in the hippocampus [$t(10)=5.008$; $P<0.0005$], striatum [$t(10)=16.394$; $P<0.0001$] and frontal cortex [$t(10)=15.107$; $P<0.0001$], respectively, when compared to control group (Table 2).

4. Discussion

SE is one of the most important medical emergencies that result in a significant alteration of neuronal function (DeLorenzo et al., 1995). SE involves enough seizure activity to induce a sustained alteration in brain function (DeLorenzo et al., 1998).

Our results for the observed behavioural alterations after pilocarpine administration match with data that were previously decribed by Marinho et al. (1997). The behavioural records can be divided into two phases. The first one occurs between 5 and 10 min after pilocarpine injection and it consists of initial akinesia, ataxic lurching and peripheral colinergic signs. The peripheral colinergic signs persist for about 20 min after the injection and at the same time, stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes) appear. The second phase occurs between 20 and 30 min after treatment and the following aspects can be observed: clonic movements of forelimbs, head bobbing and tremors. The second phase is also characterized by seizures (35–45 min) that progress in almost all of the animals to SE (1–2 h) and death in approximately 24 h.

Lipid peroxidation in a tissue is an index of irreversible biological damage of the cell membrane phospholipid, which in turn leads to inhibition of most of the sulphydryl and some nonsulphydryl enzymes (Gilbert and Sawas, 1983). Lipid peroxidation can be induced by many chemicals and in many tissue injuries, and has been suggested as a possible mechanism for the neurotoxic effects of convulsive process (Sawas and Gilbert, 1985; Walz et al., 2000). Our results demonstrated that lipid peroxidation levels are increased during acute period of pilocarpine-induced seizures.

In normal conditions, there is a steady state balance between the production of ROS and their destruction by the cellular antioxidant system. We demonstrated that the nitrite formation in the hippocampus, striatum and frontal cortex was increased after seizures (at least 45 min) and SE in adult rats, suggesting a possible increase in the ROS levels which can be involved in the neuronal damage induced by SE. However, new studies using antioxidant drugs during SE can indicate if lipid peroxidation and nitrite formation are involved in the pathophysiology of pilocarpine-induced SE.

Different receptors have been implicated in the mechanism of pilocarpine-induced seizures. Activation of M_1 muscarinic receptors consists in first step for seizure activity; serotonine, glutamate, dopamine and GABA systems appear to mediate propagation and/or maintenance of seizures (Freitas et al., 2003b; Fritschy et al., 1999).

Our results showed a down-regulation of GABAergic receptors in the different cerebral areas of adult rats that presented an increase in lipid peroxidation level during pilocarpine-induced SE. The GABA, the principal inhibitory neurotransmitter in the cerebral cortex, maintains the inhibitory tone that counterbalances neuronal excitation (Fritschy et al., 1999). When this balance is pertubed, seizures may occur (Treiman, 2001).

Excessive activation of excitatory amino acid (glutamate) receptors is believed to result in pathological increases in $[Ca^{+2}]_i$ concentration and oxidative stress, and has been implicated in many neuropathological conditions, including amyotrophic lateral sclerosis, ischemic insult, epilepsy and Parkinson's disease (Ortiz et al., 2000). Glutamatergic receptor densities had an increase in the hippocampus, striatum and frontal cortex of adult rats after pilocarpine-induced SE, suggesting that this effect in the glutamate receptor densities can be required during SE. Another study suggested that the induction of epileptogenesis in the hippocampus in pilocarpine model of epilepsy can be regulated by glutamatergic receptor (NMDA) activation that produces an elevation in $[Ca^{+2}]_i$ level and that can be responsible for aberrant changes in cerebral homeostatic mechanisms during SE induced by pilocarpine (Raza et al., 2001). Our results suggest that the increase in glutamatergic receptor density in the brain during SE can facilitate the cholinergic system stimulation with development of SRS.

Nevertheless, the role of GABA and glutamate in the development of seizure activity remains an unresolved issue. It is known that GABA and glutamate can exert anti- and proconvulsive effects in the seizures and SE induced by pilocarpine, respectively, (Fritschy et al., 1999; Costa-Lotufo et al., 2002).

It was observed that during the subsequent pilocarpine-induced SE, lipid peroxidation levels and nitrite content in hippocampus, striatum and frontal cortex were increased, suggesting possible neuronal damage, but the relation between lipid peroxidation and receptor density alterations has always been easier to propose than to demonstrate because there are certain difficulties and more studies including other areas, neurotransmitters and different receptors, as well as new experimental procedures (receptor autoradiography and/or immunohistochemistry) should be carried out to identify the essential mechanisms for generation and spread of seizures and SE induced by pilocarpine.

Acknowledgements

This work was supported by a research grant from the Brazilian National Research Council (CNPq). RMF is fellow from CNPq. The technical assistance of Maria Vilani Rodrigues Bastos and Stênio Gardel Maia are gratefully acknowledged.

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