Reactive oxygen species generated by NADPH oxidase are involved in neurodegeneration in the pilocarpine model of temporal lobe epilepsy

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ABSTRACT

Reactive oxygen species (ROS) appear to be involved in several neurodegenerative disorders. We tested the hypothesis that oxidative stress could have a role in the hippocampal neurodegeneration observed in temporal lobe epilepsy induced by pilocarpine. We first determined the spatio-temporal pattern of ROS generation, by means of detection with dihydroethidium oxidation, in the CA1 and CA3 areas and the dentate gyrus of the dorsal hippocampus during status epilepticus induced by pilocarpine. Fluoro-Jade B assays were also performed to detect degenerating neurons. ROS generation was increased in CA1, CA3 and the dentate gyrus after pilocarpine-induced seizures, which was accompanied by marked cell death. Treatment of rats with a NADPH oxidase inhibitor (apocynin) for 7 days prior to induction of status epilepticus was effective in decreasing both ROS production (by an average of 20%) and neurodegeneration (by an average of 61%). These results suggest an involvement of ROS generated by NADPH oxidase in neuronal death in the pilocarpine model of epilepsy.

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Temporal lobe epilepsy (TLE) is clinically characterized by the progressive development of spontaneous recurrent seizures from temporal lobe foci and pathologically characterized by specific morphological and cellular alterations [4], which lead to neuronal cell injury and death [9]. The hippocampus has been a focus of interest in TLE research because it contains several well-described neuronal circuits linked to seizure onset and because it develops, in the time course of the disease, a severe loss of pyramidal cells in CA1, CA3 and the dentate gyrus [20]. However, the exact cellular and molecular mechanisms by which status epilepticus induces hippocampal cell death remain to be fully understood.

As demonstrated by several studies, overproduction of reactive oxygen species (ROS), usually referred to as oxidative stress, appears to be a potential factor contributing to neuronal damage and death and it is indeed implicated in the pathogenesis of several neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis and epilepsy, among others [1,7]. ROS can be generated during normal cellular respiration and metabolic process as well as by specific enzymes such as NADPH oxidase (Nox), which generates ROS not as a byproduct, but as the primary function of the enzyme system [2]. The Nox complex is a five-subunit enzyme that transfers electrons from NADPH to molecular oxygen to produce superoxide, representing a key source of microbicidal oxidants in the immune response [6]. Despite increasing evidence for an involvement of Nox in a number of neurodegenerative disorders, still relatively little is known about its expression, regulation, and specific contribution to brain diseases [13]. Interestingly, Nox2 deficient mice exhibit neuroprotection in a MPTP model of Parkinson's disease [27] and Nox-deficient mice exhibit reduced injury after stroke [25]. In addition to unspecific drugs that affect Nox activation and expression, such as statins and angiotensin II receptor blockers, the antioxidant apocynin can specifically inhibit Nox activity by preventing translocation and assembly of the cytosolic regulatory subunits (e.g., p47phox) with catalytic membrane subunits (e.g., gp91phox) [24], which are required at least for the activation of Nox1 and Nox2 isoforms [12].

In this study, we used apocynin to evaluate the role of ROS generated by Nox in neurodegeneration that characterizes the pilocarpine model of TLE.

Male Wistar rats, weighing between 250 and 300 g, were used throughout this study. Animals were singly housed and maintained on a 12:12 h light/dark cycle. All procedures were approved by the Institutional Animal Care Committee of the Institute of Biomedical Sciences, University of São Paulo (Protocol 137/2009). All efforts were made to minimize the number of animals used and their discomfort.

Animals were randomly divided into experimental and control groups and were first injected with a low dose of the cholinergic antagonist methyl scopolamine nitrate (1 mg/kg, s.c; Sigma–Aldrich, USA) to reduce peripheral cholinergic effects. Thirty

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minutes later, animals of the experimental group received an injection of pilocarpine hydrochloride (360 mg/kg, i.p.; Sigma–Aldrich, USA) to induce status epilepticus (SE), and control animals received a similar volume of sterile saline. Approximately 30 min after pilocarpine injection, most of the animals had entered SE, which lasted 3–24 h. Diazepam (5 mg/kg, s.c., Cristália, Brazil) was administered 4 h after the onset of SE to limit behavioral seizures and to reduce mortality. The animals were sacrificed at different time points after SE onset (3, 6, 12 and 24 h) in order to permit evaluation of the time course of ROS generation (using dihydroethidium; DHE) and of neurodegeneration (by means of Fluoro-Jade B staining) in the dorsal hippocampus (between 3 and 4.5 mm posterior to the bregma). The analysis was centered in the hilus of the dentate gyrus, in the CA1a–b areas, and in the CA3b area, with minor involvement of the CA3a and CA3c zones.

DHE is a fluorescent probe which can be oxidized by superoxide and by other reactive species producing a red fluorescent precipitate, detectable by epifluorescence microscopy with a standard rhodamine filter (excitation 550 nm, emission 570 nm) [3]. The animals were quickly decapitated, and 18 μm-thick coronal sections containing the hippocampus were prepared on a cryostat. These sections were incubated with phosphate buffer (PB) containing diethylene triamine pentaacetic acid (DTPA) (100 μM) for 10 min, and then incubated with 3 μM DHE (Invitrogen, USA) in PB/DTPA for 5 min at 37 °C. For each section, digital images of DHE oxidation into the hippocampus were collected using a Nikon E1000 upright microscope coupled to a Nikon DCM1200 digital camera and analyzed in terms of integrated density by using Image J (National Institutes of Health/USA). DHE staining was evaluated within 0.04 mm^2 areas in each section and the data were normalized in relation to the control. These areas encompassed in each case a significant part of either the CA1, CA3 or hilar regions of interest, and were used to obtain data on both sides of the brain. The parameters used for capturing the digital images were kept constant, and the experiments involving control and treated animals were always run at the same time.

Rats were deeply anesthetized with ketamine hydrochloride (5 mg/100 g of body weight, i.p.) and xylazine (1 mg/100 g of body weight, i.p.), subjected to transcardiac perfusion with sterile saline and a fixative solution composed of 4% paraformaldehyde (PFA) dissolved in 0.1 M PB (pH 7.4) at 4 °C. The brains were collected, postfixed in PFA for 4 h, and transferred to a 30% sucrose solution in PB to ensure cryoprotection, which lasted for 24 h. The brain coronal sections (30 μm) were obtained on a sliding microtome adapted for cryosectioning and were processed for Fluoro-Jade B histochemistry [21]. The material was analyzed under epifluorescence microscopy to detect fluorescein (excitation 492 nm, emission 520 nm) and digital images were collected. To assess the extent of neuronal damage, the number of labeled cells/mm^2 was determined in each hippocampal region by using Image J (National Institutes of Health/USA). Measurements were taken from three sections from each animal and averaged. These sections were chosen as the most anterior, the most posterior and an intermediate section of the hippocampal area studied. Staining of sections from control and treated animals was always processed in parallel.

Animals were treated with apocynin (60 mg/L in the drinking water; daily water intake 47.4 ± 2.3 mL/animal) [14] for 7 days prior to pilocarpine injection. This represents a daily dose of about 10.4 mg/Kg. Their brains were used for the detection of intracellular ROS after SE induction by means of DHE oxidation and to evaluate the numbers of degenerating neurons by Fluoro-Jade B staining. Control animals had free access to regular water.

Values are expressed as means ± S.E.M. Statistical comparisons were performed by one-way ANOVA, followed by Tukey's multiple comparison test. Values of $p \leq 0.05$ were considered significant. The number of rats (n) used in each group is referred to in the Results

section. Statistical analyses were performed using Graphpad Prism (3.02).

DHE oxidation experiments revealed a significant increase of ROS levels in hippocampal areas of pilocarpine-injected rats (total of $n = 21$ for the different time points) in relation to control rats ($n = 7$). In the CA1 region, we observed a statistically significant increase of the fluorescence signal 6 h (ca. 16%; $n = 5$) and 24 h (ca. 15%; $n = 6$) after SE induction, whereas in the CA3 region an increase of ROS generation was only observed at 24 h (ca. 20%; $n = 6$). On the other hand, ROS generation in the hilus of the dentate gyrus was significantly increased from 3 to 24 h: ca. 11% by hour 3 ($n = 5$), 16% by hour 6 ($n = 5$), 13% by hour 12 ($n = 5$) and 15% by hour 24 ($n = 6$) after SE induction.

Degenerating neurons were not found by means of Fluoro-Jade-B staining in the control group ($n = 4$). After pilocarpine injection, Fluoro-Jade B revealed degenerating neurons in CA1 (378 ± 104 cells per mm^2) and in CA3 (203 ± 74 cells per mm^2) at 24 h after SE induction ($n = 4$), whereas in the dentate gyrus the staining was markedly increased as early as 3 h (126 ± 15 cells per mm^2, $n = 5$) after SE induction. The latter effect was sustained for all periods tested here, namely 6 h (137 ± 41 cells per mm^2, $n = 4$), 12 h (208 ± 13 cells per mm^2, $n = 4$) and 24 h (457 ± 31 cells per mm^2,
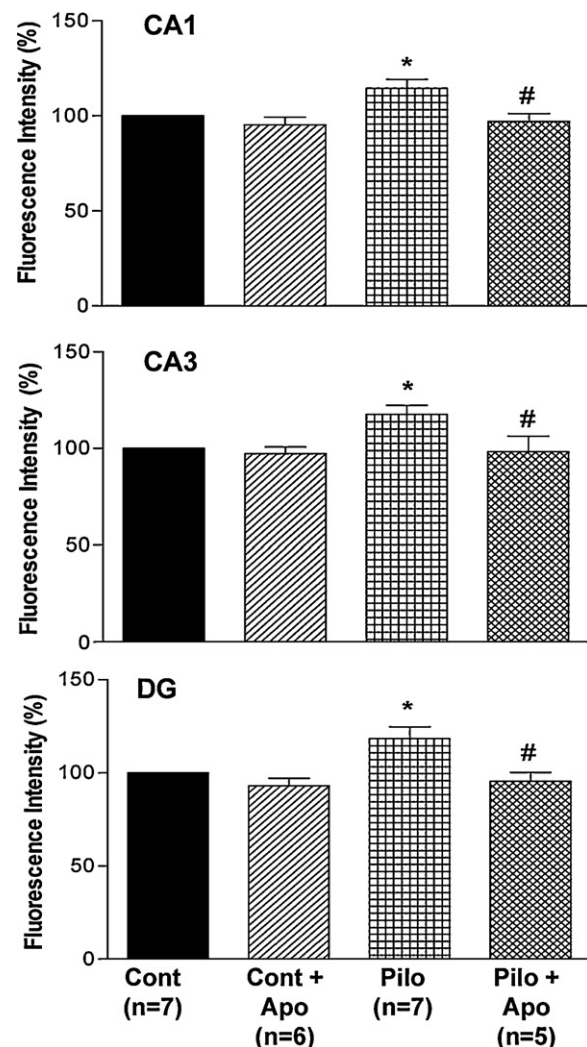
Fig. 1. Effects of apocynin treatment on ROS generation, as indicated by DHE-derived fluorescence. The graphs depict fluorescence intensity in CA1, CA3 and the hilar area of the dentate gyrus (DG) of the hippocampus from rats injected with pilocarpine (24 h after onset of SE). Cont, control; Apo, apocynin; Pilo, pilocarpine; *$p < 0.05$ Pilo vs. Cont, #$p < 0.05$ Pilo vs. Pilo + Apo.
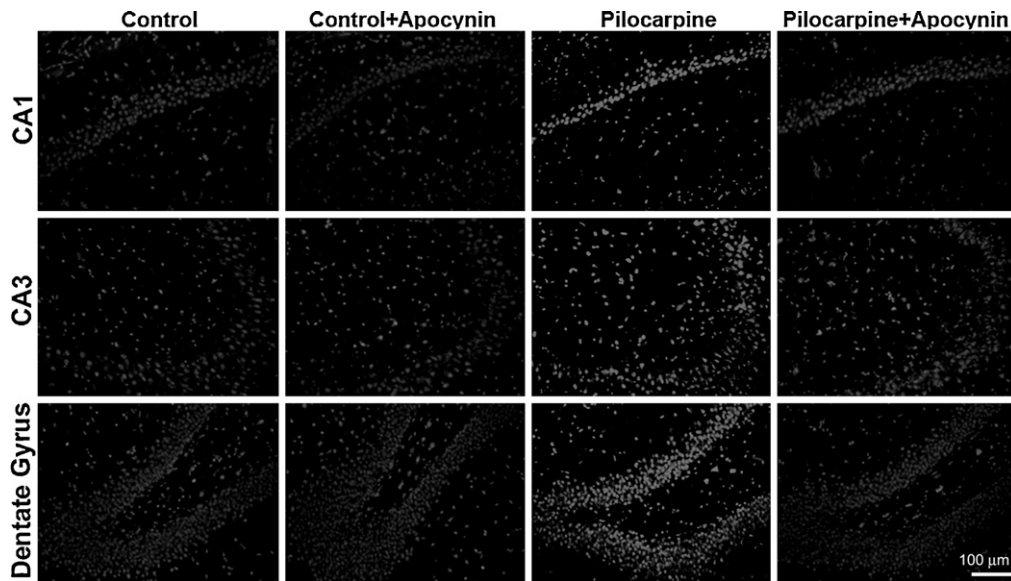
Fig. 2. Representative images taken from CA1, CA3 and hilar regions of the rat hippocampus illustrating ROS generation with and without apocynin treatment.

$n = 4$) after SE induction. It is noteworthy that labeled neurons were mainly found in the polymorphic layer of the dentate gyrus, although labeled cells were also occasionally seen in other layers, especially in granule cells.

As the time-course experiments revealed marked ROS production and neurodegeneration after 24 h of SE, we used this time point to test the effects of apocynin treatment on ROS and neurodegeneration in other groups of animals. The treatment inhibited ROS generation in the CA1 region by about 17%, in CA3 by about 20% and in the dentate gyrus by about 24% (Figs. 1 and 2). The treatment with apocynin markedly decreased the numbers of neurons stained for Fluoro-Jade B in CA1, CA3 and the dentate gyrus by about 77, 58 and 48%, respectively (Figs. 3 and 4). Numbers of rats used in the experiments with apocynin are given in Figs. 1 and 3.

The pilocarpine model has been considered one of the best models for studies of the relationship between epilepsy and the role of the hippocampus in TLE [23]. However, the molecular and cellular events responsible for the selective vulnerability of hippocampal neurons in TLE are not completely understood.

We have observed a marked hippocampal degeneration after SE induction, corroborating with data from a previous study which evidenced, by estimating the number of acidophilic neurons, early damage in dentate gyrus 3 h after the beginning of SE, followed by cell loss in CA1–CA3 regions 24 h after SE induction [10]. Another study described Fluoro-Jade C-positive neurons 4 h after SE induction and a higher number of positive cells 12 h after SE onset in CA1–CA3 pyramidal cells and in the dentate gyrus [26].In the present study ROS generation was increased in CA1, CA3 and dentate gyrus after pilocarpine-induced seizures. These results agree with several neurochemical studies that revealed that SE induces excessive production of ROS, leading to oxidative stress [5,7,8]. For instance, a significant increase of lipid peroxidation and nitrite levels has been described 6 h after pilocarpine administration, which was significantly reduced after the treatment with the liposoluble antioxidant alpha-tocopherol [22]. In the same context, vitamin E and glutathione were able to prevent the rise in lipid peroxides and hippocampal neuronal death in the kindling rat model of epilepsy, but did not prevent the development of seizures [7]. It was also demonstrated that nitric oxide production and neuronal nitric oxide synthase expression are both upregulated in the hippocampus in the lithium-pilocarpine model of epilepsy [15], representing another important source of oxidative stress after SE,
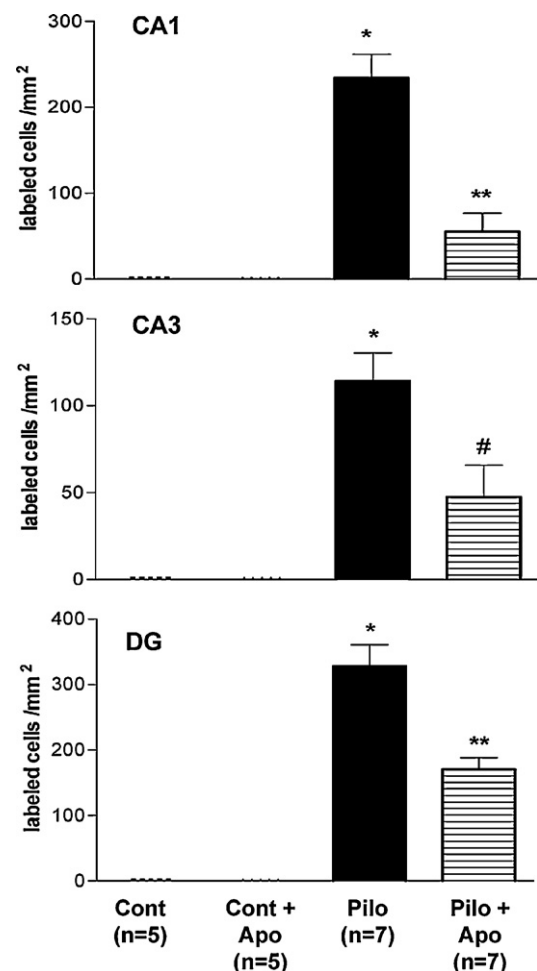

Fig. 3. Effects of apocynin treatment on neurodegeneration, as indicated by Fluoro-Jade B staining. The graphs depict cell counts of degenerating neurons in CA1, CA3 and the hilar area of the dentate gyrus (DG) of the hippocampus from rats injected with pilocarpine (24 h after onset of SE). Cont, control; Apo, apocynin; Pilo, pilocarpine; $*p < 0.001$ Pilo *vs.* Cont, $**p < 0.001$ Pilo *vs.* Pilo + Apo, $\#p < 0.05$ Pilo *vs.* Pilo + Apo.
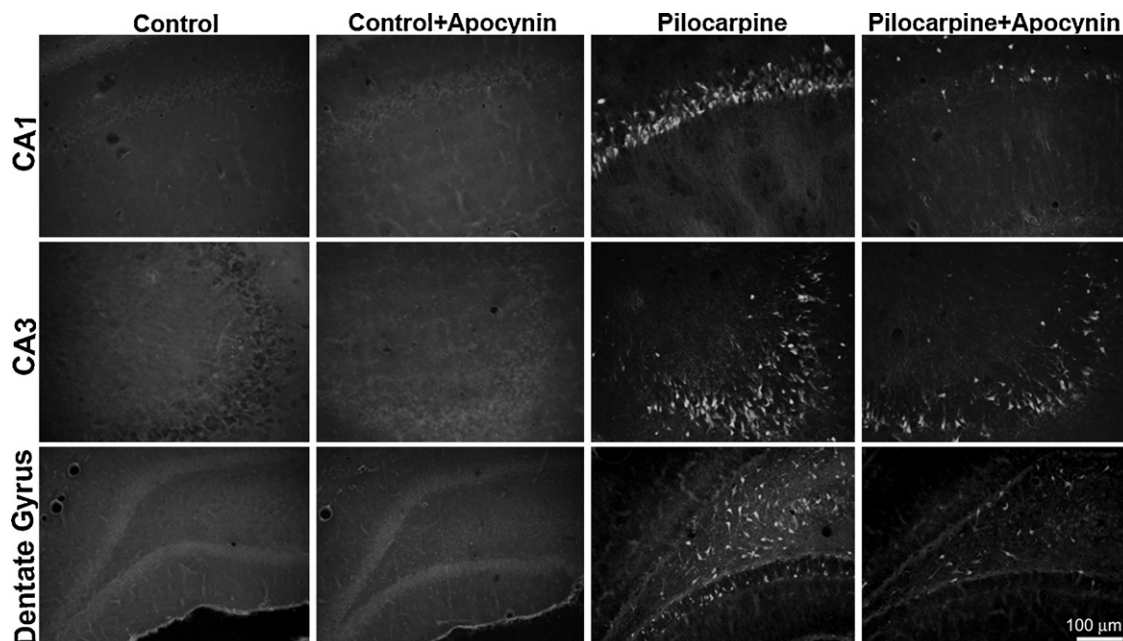
Fig. 4. Representative images taken from CA1, CA3 and hilar regions of the rat hippocampus illustrating neurodegeneration (Fluoro-Jade B staining) with and without apocynin treatment.

since nitric oxide reacts rapidly with superoxide to generate peroxinitrite [18]. Our DHE oxidation data revealed that ROS generation after SE induction coincided in general with increased neurodegeneration in CA1, CA3 and dentate gyrus. Together with the above literature data, our results support the view that ROS play a critical role in hippocampal neurodegeneration induced by pilocarpine. Moreover, our DHE oxidation assays indicated that pilocarpine was only able to slightly increase ROS production, which provide evidence that even a small change of the neuronal intracellular redox state may play a crucial role in neurodegeneration. This hypothesis is supported by the fact that ROS have a strong impact on cell homeostasis, modulating a large number of signaling cascades that can contribute to neuronal death [1].

The chronic treatment with apocynin decreased both ROS generation and neurodegeneration in the hippocampal regions analyzed here. These findings indicate that Nox-dependent oxidative stress is indeed involved in SE-induced neurodegeneration. Other studies also reported the effects of apocynin in preventing ROS generation [14,16,17] and indicated its possible role in decreasing the neurodegeneration in Huntington's and Parkinson's disease models [16,19]. However, recent studies performed on the vascular system raised controversy in that apocynin may also act as an antioxidant. Apocynin apparently requires its oxidation by H_2O_2 and myeloperoxidases before it can effectively inhibit Nox activity; otherwise, it works as an antioxidant instead of a specific inhibitor of the Nox complex [12]. Although myeloperoxidases have been described in neurons and to increase in neurodegenerative diseases [11], we cannot exclude the possibility that apocynin may also act as a direct antioxidant in the pilocarpine model of TLE.

In conclusion, the present study provided evidence that ROS generated by Nox participate in the cell death signaling that underlies hippocampal neuronal damage induced by SE. The Nox complex may then represent a promising target for therapeutic interventions in TLE, which depend on additional, intensive research on Nox inhibitors and their mechanisms of action.

Acknowledgements

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