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Activation of the phagocyte NADPH oxidase/NOX2 and myeloperoxidase in the mouse brain during pilocarpine-induced temporal lobe epilepsy and inhibition by ketamine

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Running title: Neutrophil and NOX2 in epilepsy

ABSTRACT

Excessive reactive oxygen species (ROS) production can induce tissue injury involved in a variety of neurodegenerative disorders such as neurodegeneration observed in pilocarpine-induced temporal lobe epilepsy. Ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist has beneficial effects in pilocarpine-induced temporal lobe epilepsy when administered within minutes of seizure to avoid the harmful neurological lesions induced by pilocarpine. However, the enzymes involved in ROS productions and the effect of ketamine on this process remain less documented. Here we show that during pilocarpine-induced epilepsy in mice, the expression of the phagocyte NADPH oxidase NOX2 subunits (NOX2/gp91^{phox}, p22^{phox}, and p47^{phox}) and the expression of myeloperoxidase (MPO) were dramatically increased in mice brain treated with pilocarpine. Interestingly, treatment of mice with ketamine before or after pilocarpine administration decreased this process mainly when injected before pilocarpine. Finally, our results showed that pilocarpine induced p47^{phox} phosphorylation and H_2O_2 production in mice brain and ketamine was able to inhibit these processes. Our results show that pilocarpine-induced NOX2 activation to produce ROS in mice brain and that administration of ketamine before or after the induction of temporal lobe epilepsy by pilocarpine inhibited this activation in mice brain. These results suggest a key role of the phagocyte NADPH oxidase NOX2 and MPO in epilepsy and identify a novel effect of ketamine.

Keywords: Epilepsy, Phagocytes, Neutrophils, NOX2, p47phox, Ketamine, ROS, Myeloperoxidase, Neuro-Inflammation

Introduction

Temporal lobe epilepsy (TLE) is a major neurological disease which presents a poor response to anti-epileptic drugs (AEDs) (Curia et al. 2014; Beltramini et al. 2015). To understand the pathophysiology of TLE several animal models are used such as the pilocarpine-induced temporal lobe epilepsy model. The pilocarpine model of TLE remains an animal model relevant for the human disease after 25 years from its initial characterization. Administration of the muscarinic receptor agonist pilocarpine in rats leads to repetitive limbic seizures and status epilepticus, which is followed by a latent period, the epileptogeneses phase that precedes the development of spontaneous recurrent seizures (Curia et al. 2008). Status epilepticus (SE) is one of the most serious manifestations of epilepsy. Systemic inflammation and damage of blood-brain barrier (BBB) are etiologic cofactors in the pathogenesis of pilocarpine SE while acute osmotic disruption of the BBB is sufficient to elicit seizures (Marchi et al. 2007). Epilepsy induced by pilocarpine produces several changes in variables related to the generation and elimination of oxygen free radicals in adult rats. Acute convulsions initiation in the pilocarpine model was characterized by the permeability of blood-brain barrier and vascular adhesion of leukocytes (Sills and Solomon 2009).

Ketamine is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist that can block the excitation of the NMDA receptor induced by glutamate. Several research approaches have explored the neuroprotective effect of ketamine known for its anaesthetic and co-analgesic effect. Previous studies on experimental models demonstrate that ketamine is clearly characterized by anticonvulsant properties and are able to stop status epilepticus induced by neurotoxic organophosphates (Dhote et al. 2012). Furthermore, it was reported that ketamine does not completely block the occurrence of cerebral cell damage, but can significantly reduce cognitive deficits induced during SE (Santi et al. 2001; Stewart and Persinger 2001). Therefore, ketamine also exhibits neuroprotective effects by attenuating apoptosis after traumatic brain injury (Liang et al. 2018). Ketamine strongly blocks spontaneous calcium activity in vivo (Yuryev et al. 2016). Clinical analyses in both human and animal studies also showed that

ketamine has possible immunomodulatory and anti-inflammatory effects in brain (Wang et al. 2015). Thus, ketamine has the potential to modulate inflammation. Takahashi et al. 2010 showed that ketamine exerted an anti-inflammatory effect in the presence of inflammation, and recommended that it be used in the surgery of sepsis patients. A previous study indicated that in a chronic stress-induced depression model, low dose ketamine injection showed a rapid antidepressant effect and effectively reduced the protein expression levels of IL-6, IL-1β, and TNF-α (Wang et al. 2015). Moreover, Some NMDA-receptor antagonists, such as ketamine, acts at different levels of inflammation, interacting with inflammatory cells recruitment and inflammatory mediators regulation (Loix et al. 2011). However, it was still unclear how the levels of NOX2 subunit would change in the epileptic mouse brain after ketamine administration.

Some immune cells, such as neutrophils and brain microglial cells may have an important role in the onset and development of epilepsy (Hiragi et al. 2018). Neutrophil depletion inhibited acute seizure induction and chronic spontaneous recurrent seizures. Furthermore, blood-brain barrier (BBB) leakage, which is known to enhance neuronal excitability, was induced by acute seizure activity but was prevented by blockade of leukocyte-vascular adhesion, suggesting a pathogenetic link between leukocyte-vascular interactions, BBB damage and seizure generation (Rana and Musto 2018). Consistent with the potential neutrophils involvement in epilepsy in humans, neutrophils were more abundant in brains of individuals with epilepsy than in controls (Sills et al. 2009). Activated neutrophils, macrophages and microglia produce and release large amounts of reactive oxygen species (ROS) participating to brain injury and inflammation. ROS are generated by the enzyme complex called the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) which is multi-protein, electron transport system that produces large amounts of superoxide anions via the reduction of molecular oxygen. Superoxide generates hydrogen peroxide (H_2O_2), a substrate of myeloperoxidase (MPO), an enzyme found in phagocytes and mainly in neutrophils (Hernandes and Britto 2012). NOX2-derived reactive oxygen species (ROS) and MPO are suggested to be involved in a variety of processes

of epileptogenesis and constitute an extremely deleterious event considered to be a cause and consequence of prolonged seizures (Zhang et al. 2016).

The phagocyte NADPH oxidase (NOX2) is mainly expressed in neutrophils, monocytes, macrophages, dendritic cells and microglia. NOX2, which catalyzes the reduction of molecular oxygen to form $O_2^{\bullet-}$, is a multi-subunit enzyme composed of the cytosolic proteins, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac1/2 and two membrane proteins, $gp91^{phox}$ and $p22^{phox}$. The assembly of the NOX2 complex is regulated by $p47^{phox}$ phosphorylation (El-Benna et al. 2016). In the brain, normal NOX2 function appears to be required for processes such as neuronal signaling and memory, but overproduction of oxidants contributes to neurotoxicity and neurodegeneration (Infanger et al. 2006). Indeed NOX2-derived $O_2^{\bullet-}$ and H_2O_2 production are shown in several epilepsy models (Kim et al. 2013). It has been demonstrated that microglia, the brain's phagocyte, express all components of the neutrophil NADPH complex (Patel et al. 2005; Ma et al. 2017). Abnormal NOX activity has also been suggested in Parkinson's disease, epilepsy or as a result of a stroke. Thus, it may be thought that NOX inhibitors may be useful drugs for treating diseases associated with neuronal loss.

The mechanisms contributing to brain injury in epilepsy and the protective effect of ketamine are not well-defined and the understanding of the mechanisms can lead to a novel anti-epileptogenic therapies. In the present study we examined the role of the phagocyte NADPH oxidase in epileptic pilocarpine-induced mouse model and tested the effect of ketamine after and before status epilepticus.

Materials and Methods

Reagents

Pilocarpine chlorhydrate ≥98% (titration), Ketamine hydrochloride 250mg/5ml and Atropine were obtained from Sigma-Aldrich (Germany). Triton X-100, Deoxycholate, SDS, ortho-dianisidine dihydrochloride, hydrogen peroxide and Phosphate Buffered Saline (PBS) were obtained from Sigma

Aldrich (Saint Quentin Fallavier, France). SDS–PAGE and Western blotting reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Mouse anti-gp91^{phox} and anti-p22^{phox} antibodies were supplied by Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Rabbit anti-p47^{phox}, anti-phospho-Ser328 and phospho-Ser315 antibodies were produced in our laboratory as previously described (Boussetta et al. 2010). HRP-anti-mouse and HRP-anti-rabbit antibodies were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Luminol chemiluminescence kit was obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

Animals

Thirty adult Swiss mice (25 to 30 g) from Pasteur Institute of Tunis were used. To minimize pain and discomfort for animals all measures were taken with a regular veterinary control. Animals were used along this study in accordance to Tunisian Chart on Ethics of Animal Experiments and in compliance with ethical standards. Experimental animals were allowed to adapt to laboratory conditions for at least 3 weeks before starting the experiments. They were provided with food and water ad libitum and maintained at controlled temperature (22 ± 2°C) with a 12 hours light/dark cycle. These conditions follow the recommendations reported in the "Guide for the care and use of laboratory animals" (Garber et al. 2011). The experimental protocol was approved by the Bio-Medical Ethics Committee (Pasteur Institute, Tunis) (Authorisation No.: 102/18, Ref: 2018/19/E/ NSVM).

Experimental procedure

Animals were divided into five groups of six animals:

Control group (C): Mice were injected intraperitoneally (i.p.) with saline solution NaCl: 0.9%.

Pilocarpine group (PILO): Mice received 3 injections of pilocarpine hydrochloride (100 mg/kg; i.p.) every 20 min until the beginning of status epilepticus as recommended in the literature (Gröticke et al.

2007; De Oliveira et al. 2008). To avoid side effects induced by peripheral cholinergic activation, mice were treated with atropine (1 mg/kg; subcutaneous (s.c.)) 30 min before the application of pilocarpine, to reduce cholinergic effects of pilocarpine (salivation, diarrhea and lacrimation), lessen the cholinergic component of the seizures without interfering with the development of status epilepticus and chronic seizures (Kobayashi et al. 2003; Kumar and Buckmaster 2006; Kwak et al. 2006). The doses and timing of atropine injection were determined according to the studies on mice (Fujikawa 1995; Curia et al. 2008; Lenz et al. 2017).

Ketamine group (KET): The doses of ketamine and timing of treatment were determined according to the studies on mice (Dhote et al. 2012). Mice received saline instead of pilocarpine and a subanesthetic protocol was used, it consisted in the repeated administration of ketamine (10 mg/kg; i.p.) every 30 min for a total of 3 injections. Preliminary trials confirmed that 10 mg/kg i.p. of ketamine, reported for mouse subanesthesia, and were well supported. Thus, the interval between ketamine injections was 30 min to limit the risk of overdosage. Indeed, precise data on the pharmacokinetics of ketamine after repeated i.p. administrations in mice are available (Can et al. 2016).

PILO-KET group: Mice received 3 injections of 100 mg/kg pilocarpine every 20 min until the beginning of status epilepticus. Atropine (1 mg/kg, s.c.) was administered 30 min before the application of pilocarpine. In all pilocarpine-injected mice, SE states were reached 30 min before the administration of pilocarpine. Our data are consistent with previous reports (Turski et al. 1984; Mazzuferi et al. 2012). Ketamine was injected by repeated low-doses starting 30 min after pilocarpine injection, it consisted in the administration of ketamine (10 mg/kg) every 30 min for a total of 3 injections by the intraperitoneal route. The doses and timing of treatment were determined according to the studies on the mice (Dhote et al. 2012; Mcgirr et al. 2017).

KET-PILO group: Mice received 3 injections every 30 min of 10 mg/kg ketamine 30 min prior to the 3 injections of 100 mg/kg pilocarpine every 20 min as recommended in the literature (Mazzuferi et al.

2012). Atropine (1 mg/kg, s.c.) was administered 30 min before the application of pilocarpine. Injection of ketamine 30 min prior to the injection of pilocarpine was indeed necessary to achieve protection.

All animals were visually monitored for 30 to 60 min after injection of pilocarpine and ketamine. Fifteen days after the last injection, animals were sacrificed, and their brain rapidly excised and homogenized in TBS buffer (100 mM Tris-HCl (pH=7.5), 150 mM NaCl, 2% Triton X-100, 1% Deoxycholate and 0.2% SDS) with an ultrathurax T25 homogenizator at a 2 mL/g ratio. After centrifugation at 15.000 x g for 10 min at 4°C, the supernatant was used for the determination of H_2O_2 production by luminol-amplified chemiluminescence, myeloperoxidase activity and $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, actin, phospho-ser315 and phospho-ser328.

Myeloperoxidase activity assay

Tissue samples (brain) were sonicated twice, frozen, and then centrifuged at 15.000 x g for 15 min at 4°C, and myeloperoxidase activity was assayed according to the method of Goldblum et al. 1985, the supernatant (50 μL) was mixed with phosphate buffer (350 μL) containing 0.167 mg/mL ortho-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was recorded by spectrophotometer was read every 10 s for 10 min. One unit of MPO activity was defined as that consuming 1 nmol of peroxide per minute at 22 °C. Data were expressed as difference of optical density in the first minute.

Measurement of H_2O_2 production by luminol-amplified chemiluminescence

To test the effect of ketamine on H_2O_2 production, the brain homogenate was incubated with PBS in the presence of luminol (5 μM) for 15 min at 37°C. Samples were then mixed to 5U of HRPO and chemiluminescence was evaluated with a luminometer (Auto Lumat LB953 model, EG & G Berthold) as Wymann et al. 1987 and Zappacosta et al. 2001 . Light emission was recorded in counted photons per

minute (cpm) for 15 min at 37° C (Yang et al. 2015). The percentage of inhibition was calculated in comparison to the control group.

Western blotting analysis

Proteins extracted from brain homogenate were denatured in by adding concentrated Laemmli sample buffer (30) then incubated for 2 min at 100°C. The samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) (equivalent of 25µl/well). The separated proteins were transferred to nitrocellulose membranes, and incubated with antibodies diluted in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 1% milk protein. Antibodies used were mouse anti-gp91^{phox} (1/5000) and anti-p22^{phox} (1/2000) antibodies, or primary rabbit anti-p47^{phox} (1/5000), anti-phospho-Ser328 and phospho-Ser315 (1/5000) antibodies and finally a mouse anti- β actin (1/8000) antibody at 4° C overnight under the agitator, followed by incubation with a secondary antibody HRP-anti-mouse for (phospho-Ser328, phospho-Ser315 and β-actin) and HRP-anti-rabbit for (gp91^{phox} and p22^{phox}) at the dilution (1/10000) for 1 h at 25° C. Membranes were developed using an enhanced luminol chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA). Band intensities were analyzed using Image J software. Protein levels were normalized to actin expression.

Statistical analysis

Statistical Analysis Software (SAS) studio was used for data analysis. One-way Analyses of variance (ANOVA) with post hoc Duncan tests were used to determine statistical associations between the experimental groups from MPO assay, chemiluminescence and Western blotting data. All the experimental data were expressed as means ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

Results

Myeloperoxidase (MPO) activity, a specific phagocytes marker is increased in mice brain during pilocarpine-induced epilepsy

To investigate the role of neutrophils in a model of pilocarpine-induced epilepsy, mice were injected by pilocarpine to induce epilepsy and brains were isolated to evaluate the presence of myeloperoxidase (MPO), a known marker for phagocytes such as neutrophils, monocytes, macrophages and microglia. The induction of epilepsy was assessed by clinical observation of epileptic symptoms. Results show (Table 1) that pilocarpine-induced motor seizures began with defecation, salivation, forelimb clonus, loss of posture and falling and mild body tremor that progressed during 5 to 20 min to increased levels of motor activity in all animals. The convulsive pattern presented by pilocarpine-treated mice was similar to that described by Gröticke et al. 2007 and De Oliveira et al, 2008. The injection of ketamine before or after the installation of status epilepticus by pilocarpine was able to inhibit this process, particularly, when administered before pilocarpine. Furthermore, no behavior changes were observed in mice treated only with the subanesthetic dose of ketamine (Table 1).

The presence of phagocytes in mice brain was first assessed by measuring myeloperoxidase (MPO) activity, in mice brain. Results show that a basal MPO activity was found in the brain of control mice and pilocarpine significantly increased this activity (Fig. 1). We then determined the effect of ketamine administration before and after epilepsy on brain MPO activity. Interestingly, treatment of mice with ketamine significantly decreased the enzymatic activity of MPO especially when administered before epilepsy induction. In addition, these results indicate that administration of ketamine alone has no significant effect on MPO activity in mouse brain (Fig. 1). These results suggest that the pilocarpine-induced MPO activity may be due to phagocytes (neutrophils, monocytes and macrophages) infiltration in the brain or an increase of MPO expression in microglia.

Expression of the phagocyte NADPH oxidase NOX2 is increased in mice brain during pilocarpine-induced epilepsy

To further confirm the MPO results, we analyzed the presence of the phagocyte NADPH oxidase components (gp91^{phox}/NOX2, p22^{phox} and p47^{phox}) in the brain of pilocarpine-treated mice, by using SDS-PAGE and Western blot techniques. As shown in the (Fig. 2a and 2b), the expression of the NADPH oxidase components gp91^{phox}, p22^{phox} and p47^{phox} was dramatically increased in mice brain treated with pilocarpine and ketamine reversed this expression mainly when injected before pilocarpine. These data clearly show that pilocarpine induced phagocytes infiltration in mice brain and that ketamine antagonizes this effect and suggest that NOX2 and MPO are involved in this process.

Effect of pilocarpine and ketamine on NOX2 activation in mice brain

To investigate whether the phagocyte NADPH oxidase NOX2 is activated in pilocarpine-treated mice brain we first assessed the phosphorylation of p47^{phox}, a crucial event for NADPH oxidase activation. Fig. 3 shows that pilocarpine induced p47^{phox} phosphorylation on Ser315 and Ser328 in mice brain and interestingly ketamine inhibited this phosphorylation. Western blot analysis using an antibody directed against total p47^{phox} showed that the same amount of proteins was present into each pilocarpine treated mice.

Second, as NADPH oxidase activation resulted in the production of superoxide anion which dismutates to hydrogen peroxide, a more stable reactive oxygen species molecule, we then measured hydrogen peroxide in mice brain. Results show that pilocarpine induced a significant increase of H_2O_2 in mice brain. Treatment with ketamine before or after pilocarpine reduced the pilocarpine-induced H_2O_2 production. Although, this reduction is important, when ketamine was injected before pilocarpine. However, repeated ketamine administration in healthy mice alone had no significant effect on H_2O_2 production (Fig. 4). These data clearly show that pilocarpine induces p47^{phox} phosphorylation, NADPH

oxidase activation and ROS production in mice brain and that ketamine was able to inhibit this activation. Particularly, when injected before the induction of epilepsy by pilocarpine in mice.

Discussion

The present study shows that NADPH oxidase NOX2 is activated and MPO activity is increased in pilocarpine-induced epileptic mouse model and that ketamine was able to inhibit the process. Epilepsy is characterized by an enduring predisposition to generate seizures and its development is accompanied by alterations in many cellular and molecular processes. The enormous complexity of the human nervous system represents a barrier for modern drug discovery. Moreover, comprehension of the complex mechanisms underlying epileptogenesis and seizure generation in temporal lobe epilepsy cannot be fully acquired in clinical studies with humans. As a result, the use of appropriate animal models is essential. The animal models of epilepsy, such as the model of pilocarpine, are useful for studying the relationship between epilepsy and NADPH oxidase dysfunctions. We propose that the pilocarpine model can be a valuable tool to investigate the mechanisms involved in temporal lobe epilepsy (TLE). Our protocol involves repeated 3 injections of small doses of pilocarpine 100 mg/kg every 20 min until the installation of status epilepticus, which appears to be effective in mice (Gröticke et al. 2007; Mazzuferi et al, 2012). Mice were administered 1 mg/kg muscarinic cholinergic antagonist atropine. Administration of atropine (30 min prior to pilocarpine) has been used to block the peripheral cholinergic side effects of pilocarpine (Curia et al. 2008; Fujikawa 1995), without interfering with the development of status epilepticus and chronic seizures (Kobayashi et al. 2003; Kumar and Buckmaster 2006; Kwak et al. 2006). According to the literature, pilocarpine can induce the secretion of saliva, accompanied by the hypersecretion of bronchial mucus, which can lead to transient inhibition of the respiratory system or apnea. To prevent this adverse effect, anticholinergic drugs, such as atropine, can be administered to prevent pilocarpine from binding to its peripheral receptors (Lenz et al. 2017).

Pilocarpine produced a sequence of behavioral alterations including staring spells, limbic gustatory automatisms and motor limbic seizures that developed over 5-20 min. Our data are consistent with previous reports (Turski et al. 1984; Pitsch et al. 2007; Müller et al. 2009; Mazzuferi et al. 2012). The ketamine has often been used as a protective agent in many types of traumatic injury (Liang et al. 2018), although its anti-inflammatory effect and its effect on neutrophil functions is not yet fully understood (Dhote et al. 2012; Losset al. 2012).

Previous studies in mice, showed that after an injection of ketamine (10 mg/kg, i.p.), the concentration peak in brain tissue (7.03 nmol/g) was observed 10 minutes after ketamine administration and then declined to 0.09 nmol/g in the 240 minute samples (Can et al. 2016). So, there is evidence that repeated administration of subanesthetic doses of ketamine may have beneficial long-term effects. Previous studies showed that repeated subanesthetic ketamine has been shown to improve clinical outcomes for treatment resistant depression (Rasmussen et al. 2013; Loo et al. 2016; Cusin et al. 2017). Repeated ketamine administration has also been associated with attenuation of the acute ketamine-induced dissociation, derealization, and dizziness over time (Singh et al. 2016a; Zanos et al. 2018). With this knowledge, we injected ketamine at 10 mg/kg, is a subanesthetic dose, every 30 minutes. Manocha et al. 2001 shows the anticonvulsant effect of ketamine associated with other drug like (GABA, diazepam and baclofen) before the installation of epilepsy in mice. This study was carried out during the phase of epileptogenesis "latency phase" which precedes the recurrent seizures of epilepsies. According to a study based on continuous video-EEG recordings, observed that rats that had had pilocarpine-induced SE developed recurrent spontaneous seizures (RSSs) after a mean latency of 14-15 days (Cavalheiro et al. 1991). To study, the effect of ketamine on NOX2 activation during the latent period before the intervention of central immune cells such as microglia. However, a study reported that SE provoked time-dependent changes in the microglial morphology. Furthermore, a significant increase in the number of rod-shaped cells, an active form of microglial, was only evident in the brain at 2 weeks after SE. For these reasons, the mice were sacrificed 15 days after treatment.

Previous studies on the immune system in the epileptic brain indicating that the infiltration during epileptogenesis of peripheral immune cells such as neutrophils may contribute to the development of chronic epilepsy and recurrence of seizures (Vinet et al. 2016). The researchers found that microglia showed weak immune activation, in contrast to infiltrated peripheral immune cells that showed a strong immune response. Both cell types expressed high levels of phagocytosis markers after 2 weeks of SE. Also, Giordano et al. 2015 show that no morphological changes indicative of microglial activation were observed by Iba-1 immunohistochemistry in any considered brain region during epileptogenesis. Overall, studies indicate that neutrophils could play a detrimental role during epileptogenesis compared to microglia. Because of this, microglia are generally considered to play a pro-epileptogenic role. However, infiltration of peripheral immune cells during epileptogenesis such as leukocytes, granulocytes and monocytes/macrophages might also contribute to the development of chronic epilepsy and recurrent seizures. The pilocarpine mouse model of TLE, leading to local inflammation, blood-brain barrier disruption, and infiltration of leukocytes, as well as sustained endothelial and microglial cell activation in areas of neurodegeneration. The importance for epileptogenesis of proinflammatory signaling cascades, as well as leukocyte–endothelium interactions and BBB leakage, has been demonstrated in the pilocarpine model of TLE (Ravizza et al. 2008). Thus, Pilocarpine caused acute peripheral pro-inflammatory changes leading to blood-brain barrier (BBB) leakage prior to SE (Marchi et al. 2007b). However, comparison of *in vivo* effects of pilocarpine pointed to a role of systemic inflammation in ictogenesis (Marchi et al. 2007), possibly because of activation of muscarinic receptors on leukocytes. Furthermore, neutrophils are also involved, in the process of inflammation in the brain during epilepsy (Webster et al. 2017), underscoring the importance of systemic inflammation for ictogenesis. Silverberg and colleagues showed that brief episodes of tonic-clonic seizure activity are sufficient to trigger lymphocyte infiltration into the neocortex and hippocampus (Silverberg et al. 2010). The NADPH oxidase is a multi-subunit enzyme, expressed in phagocytes that catalyzes the reduction of molecular oxygen and oxidation of NADPH to generate

superoxide radicals ($O_2^{\bullet}$). The subunits localize in both membrane-bound (cytochrome b558, comprised of $p22^{phox}$ and $gp91^{phox}$) and cytoplasmic ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) locations. Upon stimulation, activation of a low-molecular weight G protein (Rac or Rac2) and phosphorylation of $p47^{phox}$ initiates migration of the cytosolic elements to the plasma membrane, whereby a functional complex forms that generates $O_2^{\bullet}$.

Several mechanisms are described that participates to the anti-inflammatory effects of ketamine. For example, ketamine interacts directly with some ion channels such as the large conductance Ca^{++} activated K^+ channels. Blockade of these channels is demonstrated to prevent the inflammatory signals arising from the periphery to activate the microglia by suppressing the pro-inflammatory cytokines production of the immune cells in the CNS. The dose used in this experimentation was, however, particularly high (Hayashi et al. 2011). Moreover, Weigand et al. 2000 indicate that ketamine can attenuate important pro-inflammatory key functions of stimulated neutrophils in vitro. To which extent these mechanisms account for the inflammatory "protective" effects of ketamine remains to be elucidated. The mechanisms of regulation of brain NADPH oxidase complex by ketamine during the epileptogenesis phase remain unknown; while several data sources indicate the importance of Ca^{++} and protein kinases in the production of ROS (Görlach et al. 2015) because of the overactivity of NADPH oxidase causes the production of ROS in the brain (Qin et al. 2018; Hervera et al. 2018). General anesthetics, because of their direct effects on glutamatergic and GABAergic transmission, have a neuroprotective action that has been demonstrated in animals (Degos et al. 2013). In addition, some molecules used in anesthesia, such as thiopental, midazolam or ketamine, have peripheral immunomodulatory effects in vivo and in vitro (Colucci and Puig 2013). They are able to inhibit the main mechanisms involved in the immune response such as neutrophil adhesion, phagocytosis and the release of free radicals (Nishina et al. 1998).

To study the role of phagocytes such as neutrophils monocytes and microglia in a model of pilocarpine-induced epilepsy, mice were injected with pilocarpine, the brain was isolated, and

myeloperoxidase (MPO) activity was evaluated. It uses H_2O_2 as substrate and generates oxidizing species such as hypochlorite. In this study, we showed an increase in MPO activity in the brain of epileptic mice during epileptogenesis. Furthermore, treatment of mice with ketamine, whether before or after the induction of epilepsy; induces a reduction in the activity of this inflammatory mediator (MPO). Although this important reduction, when administered before of induction of epilepsy by pilocarpine. Several studies show that, during epileptogenesis, oxidative stress increases considerably (Waldbaum and Patel 2010). In addition, increased concentrations of hydrogen peroxide, an essential substrate for MPO, in the brains of mice during epileptogenesis (Bellissimo et al. 2001). Recently, it has been shown that MPO is increased in the brain of a patient with refractory epilepsy, as well as in a mouse model of epilepsy (Zhang et al.2016). One of the oxidants released by activated phagocytes is hypochlorous acid formed via the myeloperoxidase (MPO)-H2O2-Cl (-) (Üllen et al. 2013). So the enhanced level of MPO activity is one of the best diagnostic tool of inflammatory and oxidative stress biomarkers among these commonly occurring diseases in brain (Khan et al. 2018).

In this study, we show that expression of NADPH oxidase subunits was increased in epileptic mice. This increase could be due to recruited phagocytes at the brain or un increase of NOX2 expression in residual neurons and microglia (Pestana et al. 2010). In addition, simultaneous treatment of ketamine inhibits the increased activity of NADPH oxidase in the brain of epileptic mice. This decrease is more significantly appreciated in the batch pretreated with ketamine than in the batch posttreatment with ketamine during the epileptogenesis phase. These results suggest that ketamine inhibited phagocyte NADPH oxidase activity when administered before of induction of epilepsy by pilocarpine. It is therefore possible that pretreatment of ketamine exerts its neuroprotective effects in preventing oxidative stress caused by hyper-excitability. Moreover, treatment with subanesthetic doses of ketamine, an NMDA receptor antagonist, may be mediated by mechanisms related to enhancement of glutamatergic neurotransmission and calcium-channel (Honeycutt and Chrobak 2018). As well suppression of oxidative damage induced by NADPH oxidase (Brennan-Minnella et al. 2013).

To investigate whether NOX2 is activated in pilocarpine-treated mice, we measured hydrogen peroxide production, a more stable ROS generated from superoxide anion, a direct product of NOX2. The present study demonstrates that epilepsy induces an increase in H_2O_2 production in the brain. One previous study suggested an involvement of NADPH oxidase generated ROS in neuronal death after TLE (Pestana et al. 2010). In addition, recent evidence suggests that hydrogen peroxide derived from NADPH oxidase contributes to the oxidative stress generation in brain. As phosphorylation of $p47^{phox}$ is a crucial event for NADPH oxidase activation, we next examined the effect of pilocarpine and ketamine on this process. In this study, we showed an increase the phosphorylation of $p47^{phox}$ on Ser-315 and Ser-328 in the brain of epileptic mice by pilocarpine during epileptogenesis. Our work shows that treatment with ketamine inhibits significantly the phosphorylation of $p47^{phox}$ on Ser-315 and Ser-328, an essential process for the activation of NADPH oxidase. These two serine residues are located in a PKC consensus phosphorylation site (El-benna et al. 2009), whereas ketamine indirectly inhibits the phosphorylation of PKC (Réus et al. 2011). In addition to their ability to phosphorylate $p47^{phox}$ and PKC, they have been shown to induce the regulation of NADPH oxidase activation in brain (Bedard and Krause 2007). Although, the mechanism of regulation of this ketamine-targeted enzyme complex remains to be determined, we can conclude that ketamine probably prevents the activation of NADPH oxidase by altering the phosphorylation of $p47^{phox}$ dependent on Ca^{++} and PKC, at least on serine 315 and 328. Ketamine, because of its anti-glutamatergic and anti-inflammatory properties, could represent a possible therapeutic pathway for the management of cerebral affections, especially when they are the consequence of epilepsy.

The present study provides evidence that pilocarpine induces NOX2 and MPO activation in mice brain and ketamine inhibits this process. Novel strategies targeting NOX2 and MPO could represent promising approaches for therapeutic intervention in epilepsy.

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Disclosure

The authors report no conflicts of interest in this work.

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Figure Legends

Figure 1. Effects of pilocarpine and ketamine administration on the presence of myeloperoxidase activity in mice brain. Mice were injected with pilocarpine alone or with ketamine, sacrified and the brains homogenized as described in the method section. MPO activity was then measured using H_2O_2 and orthodianisidine. The results are expressed as the mean ± SEM (n = 6). With $p<0.05$ were considered significant (*) indicated significance for ketamine or pilocarpine vs. control and (§) for KET-PILO and PILO-KET vs. P. (**) and (§§) indicated $p<0.01$.

Figure 2. Effects of pilocarpine and ketamine administration on the presence on the phagocyte NADPH oxidase subunits in mice brain. Mice were injected with pilocarpine alone or with ketamine, sacrified and the brains homogenized as described in the method section. Proteins were denaturated and analyzed by SDS-PAGE and western blots. Western blot analysis (a) and densitometric quantification (b) of the $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$ and actin of brain in the different groups of mice. The results are expressed as the mean ± SEM (n = 6). With $p<0.05$ were considered significant (*) indicated

significance for ketamine or pilocarpine vs. control and (§) for KET-PILO and PILO-KET vs. P. (**) and (§§) indicated $p<0.01$.

Figure 3. Effects of pilocarpine and ketamine administration on the phosphorylation of p47^{phox} in mice brain. Mice were injected with pilocarpine alone or with ketamine, sacrified and the brains homogenized as described in the method section. The proteins were denatured by adding 5X concentrated modified Laemmli sample buffer. After denaturation, the tissue homogenates of brain (eq. of 25µl/well) were subjected to SDS-PAGE (10 %) and analyzed by western blotting using specific rabbit anti-phospho-Ser315-p47^{phox} and anti-phospho-Ser328-p47^{phox} polyclonal antibodies. The blots were reprobed with rabbit anti-p47^{phox} antibody as loading control (Figure 3.a). The ratio of phospho-p47^{phox} to the total amount of p47^{phox} was quantified using Image J 1.43u software. Values are expressed as means ± SEM of 6 independent experiments. With $p<0.05$ were considered significant (*) indicated significance for ketamine or pilocarpine vs. control and (§) for KET-PILO and PILO-KET vs. P. (**) and (§§) indicated $p<0.01$.

Figure 4. Pilocarpine induced H_2O_2 production in mice brain and ketamine inhibited this effect. Mice were injected with pilocarpine alone or with ketamine, sacrified and the brains homogenized as described in the method section. H_2O_2 was then measured using chemiluminescence. The results are expressed as the mean ± SEM (n = 6). With $p<0.05$ were considered significant (*) indicated significance for ketamine or pilocarpine vs. control and (§) for KET-PILO and PILO-KET vs. P. (**) and (§§) indicated $p<0.01$.

Table 1. Effects of ketamine on clinical symptoms during of TLE.

Group	Control (C)	Pilocarpine (PILO)	Ketamine (KET)	(PILO-KET)	(KET-PILO)
30 to 60 minutes after the last injection					
Behavior Changes	**No behavior changes**	**Increase of (compared to controls):** -Intensity of oro-facial automatisms **(++++)** -Involuntary muscle contraction **(++++)** -Clonies of forelegs **(++++)** -Hypersalivation **(++++)** -Mâchonnements **(++++)** -Vibrios movements **(++++)** -Nods of the head **(++++)** -Whiskers movements **(++++)**	**No behavior changes**	**Decrease of (compared to pilocarpine):** -Intensity of oro-facial automatisms **(++)** -Involuntary muscle contraction **(++)** -Clonies of the forelegs **(++)** -Hypersalivation **(++)** -Mâchonnements **(++)** -Vibrios movements **(++)** -Nods of the head **(++)** -Whiskers movements **(++)**	**Decrease of (compared to pilocarpine):** -Intensity of oro-facial automatisms **(+)** -Involuntary muscle contraction **(+)** -Clonies of the forelegs **(+)** -Hypersalivation **(+)** -Mâchonnements **(+)** -Vibrios movements **(+)** -Nods of the head **(+)** -Whiskers movements **(+)**

All animals were visually monitored for 30 to 60 min after injection of pilocarpine and ketamine. (++++): Very important (++): average (+): Low

1

Figure 1 (Tannich et al)

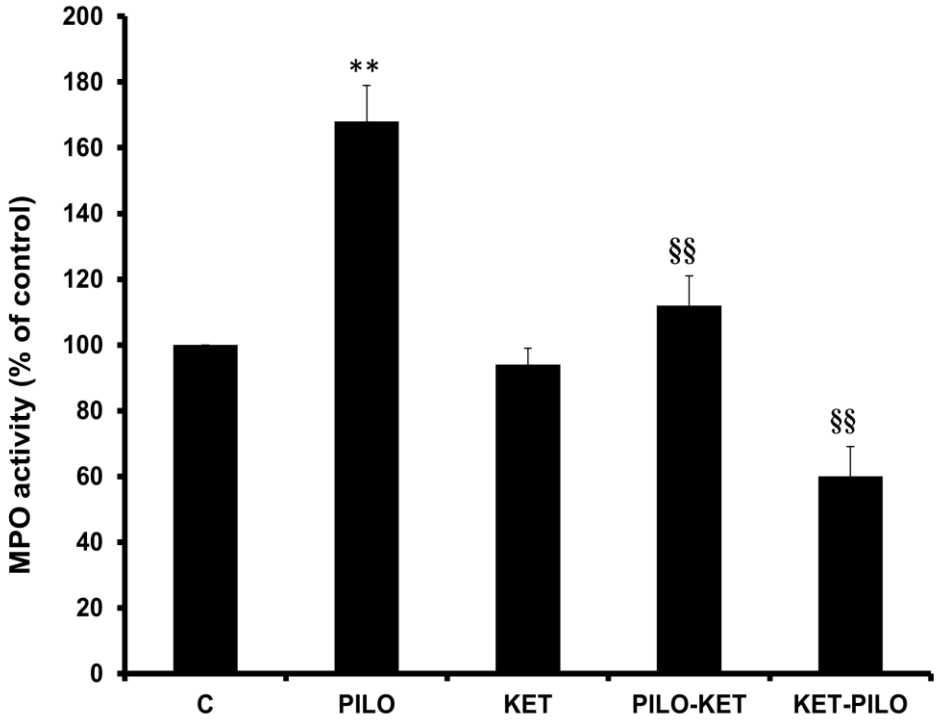


Figure 2 (Tannich et al)

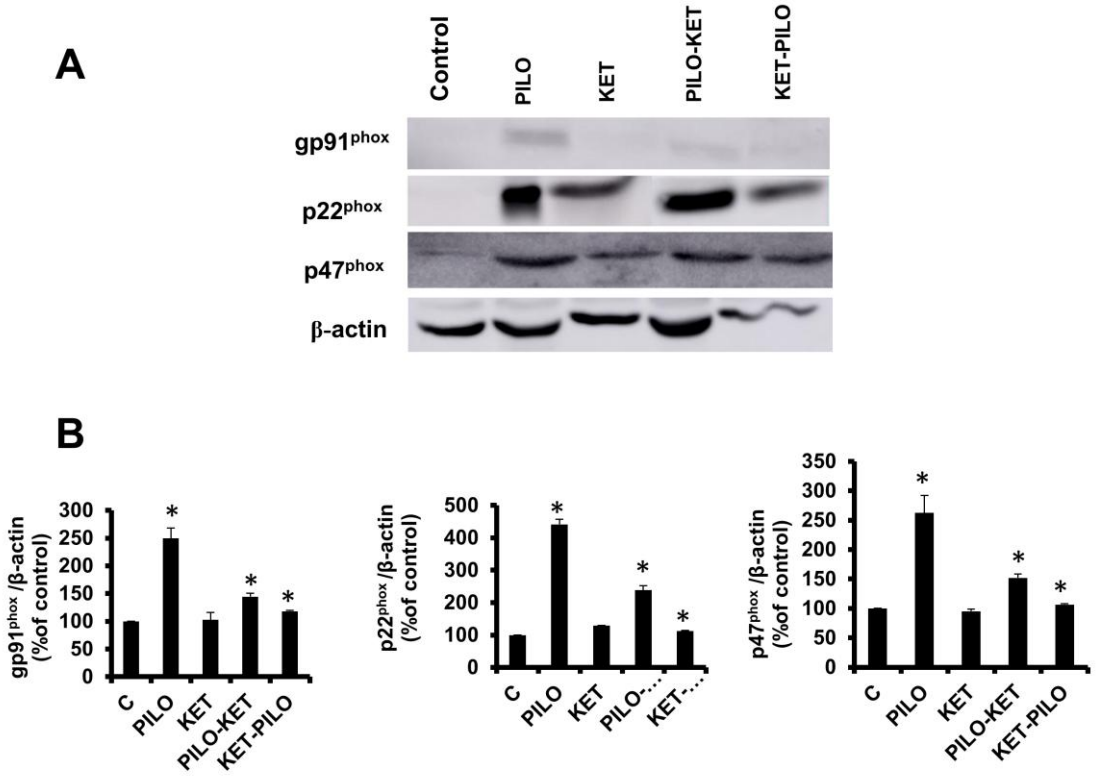
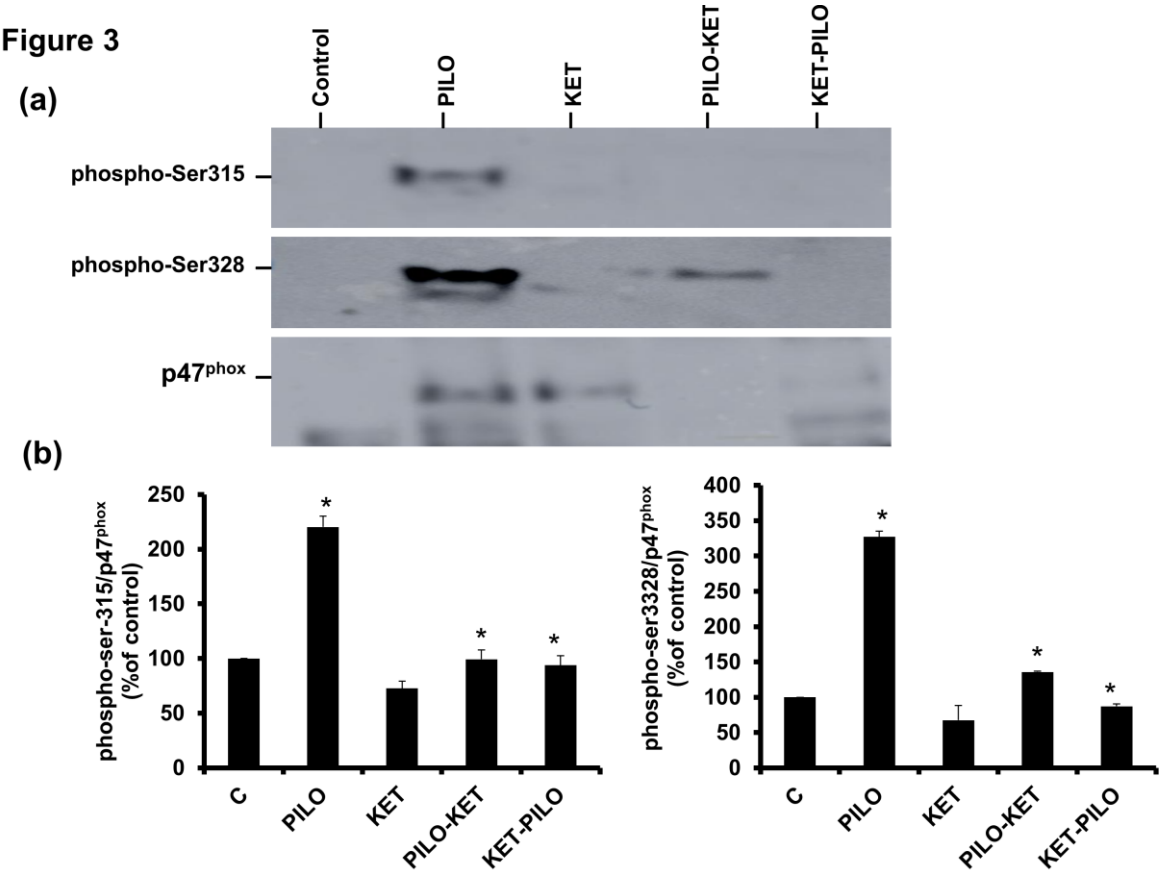


Figure 3


(a)
Control
PILO
KET
PILO-KET
KET-PILO
phospho-Ser315
phospho-Ser328
p47phox
(b)
phospho-ser-315/p47phox (%of control)
250
200
150
100
50
0
C
PILO
KET
PILO-KET
KET-PILO
phospho-ser3328/p47phox (%of control)
400
350
300
250
200
150
100
50
0
C
PILO
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PILO-KET
KET-PILO
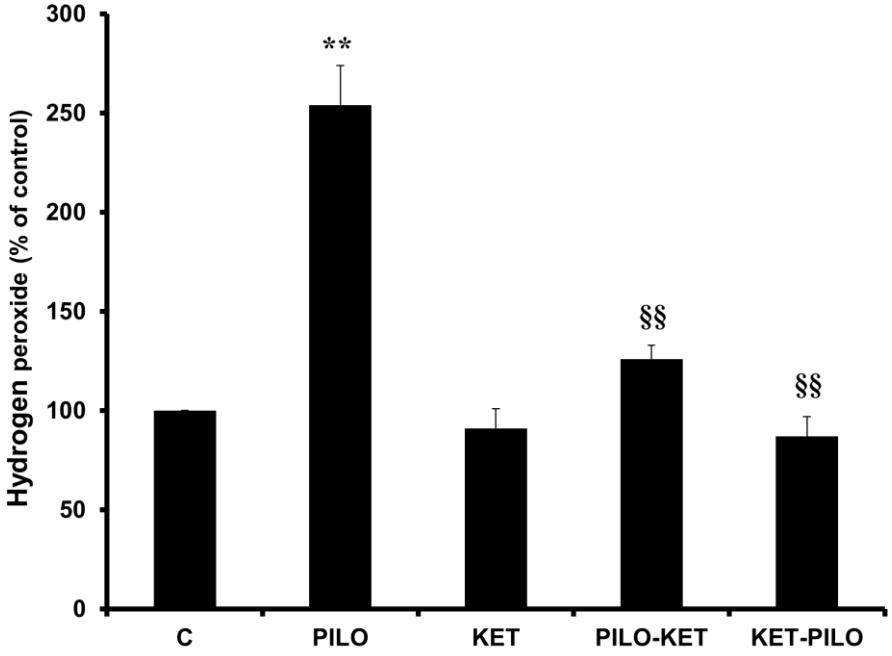
Figure 4 (Tannich et al)