Different sub-anesthetic doses of ketamine increase oxidative stress in the brain of rats

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A B S T R A C T

Schizophrenia is a complex neuropsychiatric disorder in which symptoms can be classified as either positive, such as delusions and hallucinations, or negative, such as blunted affect and social withdrawal. However, the mechanisms underlying this disease are poorly understood. There is evidence that reactive oxygen species (ROS) play an important role in the pathogenesis of many diseases, particularly those which are neurological and psychiatric in nature. Ketamine has been used to induce a schizophrenia-like condition as an animal model in which to study this condition. In the present study we tested the effects of sub-anesthetic doses of ketamine on various parameters of oxidative stress in the brain of rats. Our results indicate that lipid peroxidation and tissue protein oxidation were affected by varying sub-anesthetic doses of ketamine in multiple cerebral structures. Additionally, the activity of the antioxidant enzymes CAT and SOD was measured and was also found to be altered in most of the structures tested. In conclusion, we observe an increase in oxidative damage marked by an increase in lipid peroxidation, oxidative protein damage and a decrease in enzymatic defenses, in an animal model of schizophrenia. Given that oxidative stress could be related to schizophrenia, these findings may explain, at least in part, the mechanisms underlying in this disease.

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1. Introduction

Schizophrenia is a disease defined by a constellation of symptoms that can differ across individuals with respect to their presence, frequency, severity, and topography. This heterogeneity of symptoms has complicated the search for the etiology of the disease as well as for the means of its treatment (Bowie and Harvey, 2006). Because of the pervasiveness of associated deficits and the fact that it frequently runs a life-long course, it is among the top ten leading causes of disease-related disability in the world (Tandon et al., 2008).

The signs and symptoms of schizophrenia include alterations in aspects of consciousness, awareness, orientation, and perception, as well as cognitive impairments that affect thought and language, and neurological soft signs (Bowie and Harvey, 2006). According to DSM-IV criteria (Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition), the symptoms of schizophrenia are classified as positive symptoms, such as delusions and hallucinations, and negative symptoms, such as blunted affect and social withdrawal.

The mechanisms of this disease are poorly understood. A dysfunction of the dopaminergic system has been postulated to be a contributing factor based in part on the functional roles of dopamine with respect to physiological and illness-associated cognitive performance, especially working memory, as well as reward circuitry (Meisenzahl et al., 2007). This hypothesis was widely accepted for decades based on the observations that the symptoms caused by the abuse of stimulants (such as amphetamines and cocaine) resemble the positive symptoms of schizophrenia and that the antipsychotic drugs used to treat the disorder, such as haloperidol and chlorpromazine, have in common an ability to block dopamine D2 receptors. The binding affinity of the receptors for these drugs correlates in a highly significant fashion to their clinical potency in ameliorating psychosis (Tsai and Coyle, 2002). A recent study reported abnormalities in glutamate receptors, suggesting that an interaction between different neurotransmitter systems may also be important (Goff and Coyle, 2001). Typically, antipsychotic medications that block dopamine D2 receptors are effective in treating the psychosis associated with schizophrenia but have limited effects on the negative symptoms and cognitive impairments. Considerable research has demonstrated that noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists, such as the dissociative anesthetics phencyclidine and ketamine, reproduce the cardinal symptomatic features of schizophrenia (Tsai and Coyle, 2002).
Many animal models have been utilized to try to understand this complex disease; however the lack of a clear genetic underpinning has made drug-induced psychomimetic models particularly important (AU1). In humans, ketamine can produce hallucinations and paranoia similar to the positive symptoms of schizophrenia (Hunt et al., 2006). Ketamine can also produce social withdrawal, poverty of speech, and blunted affect resembling the negative symptoms of the disease (Adler et al., 1999; Krystal et al., 1994). Because of these properties, ketamine has been used to induce a schizophrenia-like condition as an animal model in which to study this condition.

Considerable research has shown that reactive oxygen species (ROS) have an important role in the pathogenesis of many diseases, especially in neurological and psychiatric diseases (Takuma et al., 2004). Oxidative stress may be a common pathogenic mechanism underlying many major psychiatric disorders as the brain has comparatively greater vulnerability to oxidative damage (Ng et al., 2008). In this context, it has already been reported that free radicals are elevated in patients diagnosed with schizophrenia (Lohr and Browning, 1995; Mahadik and Mukherjee, 1996; Reddy and Yao, 1996; Yao et al., 1998, Fendri et al., 2006). More recently, Young et al. (2007) reported that there was an increase in protein carbonyl groups as well as damage to the DNA in schizophrenic patients, suggesting that an oxidative alteration had occurred. Data from the literature shows that activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase were decreased and levels of malondialdehyde were elevated in patients with all forms of schizophrenia studied (Ng et al., 2008). In this context, it has already been reported that free radicals are elevated in patients diagnosed with schizophrenia (Lohr and Browning, 1995; Mahadik and Mukherjee, 1996; Reddy and Yao, 1996; Yao et al., 1998, Fendri et al., 2006). More recently, Young et al. (2007) reported that there was an increase in protein carbonyl groups as well as damage to the DNA in schizophrenic patients, suggesting that an oxidative alteration had occurred. Data from the literature shows that activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase were decreased and levels of malondialdehyde were elevated in patients with all forms of schizophrenia studied as compared to normal controls (Zhang et al., 2006).

Given that oxidative stress in the brain has been proposed to play a role in the etiology of neuropsychiatric disorders, in the present study we examined the effects of sub-anesthetic doses of ketamine on parameters of oxidative stress such as thiobarbituric acid-reactive substances (TBARS), carbonyl and sulfhydryl content of protein, and catalase (CAT) and SOD activities in the brain of rats.

2. Materials and methods

2.1. Animals

Male adult (90-day-old) Wistar rats weighting 300 g were obtained from our breeding colony. The animals were housed five to a cage with food and water available ad libitum and were maintained on a normal 12-h light/dark cycle (lights on at 7:00 AM). This study was performed in accordance with the Brazilian Society for Neuroscience and Behavior (SBNect) recommendations for animal care and with the approval of Ethics Committee from Universidade do Extremo Sul Catarinense.

2.2. Acute administration of ketamine

A single injection of sub-anesthetic doses of ketamine (4, 10 or 30 mg/kg, intraperitoneal) or saline (0.9%) was given to rats, and, 30 min after administration, the animals were killed by decapitation, the brain was removed, and the cerebellum, prefrontal cortex, hippocampus, striatum and cerebral cortex were obtained.

2.3. Tissue preparation

Brain structures were homogenized according to the buffer requirements of each assay. For total thiol and carbonyl content determinations, the striatum was homogenized with 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. For measurement of the antioxidant enzymes CAT, the homogenate was dissolved in 20 mM potassium phosphate buffer, pH 7.0, and for SOD activity assays, in 50 mM Tris–HCl buffer containing 1.0 mM EDTA, pH 8.2. Homogenates were centrifuged at 750 g for 10 min at 4 °C. The pellet was discarded, and the supernatant was immediately separated and used for the measurements.

2.4. TBARS assay

TBARS is a measurement of lipid peroxidation and was determined as described by Ohkawa et al. (1979). Briefly, homogenates in 1.15% KCl were mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS was determined by the absorbance at 535 nm. The results were reported as nmol of malonaldehyde per mg of protein (nmol/mg protein).

2.5. Protein carbonyl content

Oxidatively modified proteins present an enrichment of carbonyl content (Stadtman, 1990). In this study, carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm. Briefly, 100 ml of homogenate were added to plastic tubes containing 400 ml of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 h and vortexed each 15 min. After that, 500 ml of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 1000 g for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 ml ethanol:ethyl acetate (1:1, v/v), vortexed, and centrifuged at 1000 g for 3 min. This washing procedure was repeated once more and, after centrifugation, the supernatant was discarded and the pellet resuspended in 600 ml of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60 °C for 15 min. After that, it was centrifuged at 1000 g for 3 min and the absorbance was measured at 370 nm (UV) in a quartz cuvette. Results were reported as nmol of carbonyl content per mg of protein (nmol/mg protein).

2.6. Total thiol content

This assay is based on the reduction of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 50 ml of homogenate was added to 1 ml of PBS buffer pH 7.4 containing 1 mM EDTA. Then 30 ml of 10 mM DTNB, prepared in a 0.2 M potassium phosphate solution pH 8.0, was added. Subsequently, the mixture was incubated for 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Results were reported as nmol of TNB per mg of protein (nmol/mg protein).

2.7. Superoxide dismutase assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O2−; a substrate for SOD (Marklund, 1985). The inhibition of autoxidation of this compound thus occurs when SOD is present, and the enzymatic activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double beam spectrophotometer with temperature control. A calibration curve was performed using purified SOD as the standard, in order to calculate the specific activity of SOD present in the samples. A 50% inhibition of pyrogallol autoxidation is defined as 1 unit of SOD, and the specific activity is represented as units per mg of protein.

2.8. Catalase assay

CAT activity was assayed using a double-beam spectrophotometer with temperature control. This method is based on the disappearance of H2O2 at 240 nm in a reaction medium containing 20 mM H2O2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1-0.3 mg protein/ml (Aebi, 1984). One CAT unit is defined as 1 mol of
hydrogen peroxide consumed per minute, and the specific activity is reported as units per mg protein.

2.9. Protein determination

Protein was measured by the Lowry (1951) method using bovine serum albumin as a standard.

2.10. Statistical analysis

Data were analyzed by the Student’s t-test or by one-way analysis of variance (ANOVA) followed by the Tukey test when the F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A value of $p<0.05$ was considered to be significant.

3. Results

In this study, we investigated whether lipid peroxidation, as determined by assaying TBARS, and tissue protein oxidation, as measured by both sulfhydryl and carbonyl content, were affected by different sub-anesthetic doses of ketamine. Fig. 1 shows that TBARS increases significantly in the cerebellum [$F_{(3,12)} = 9.250$; $p = 0.007$], pre-frontal cortex [$F_{(3,12)} = 15.787$; $p = 0.024$], hippocampus [$F_{(3,14)} = 6.365$; $p = 0.003$], striatum [$F_{(3,16)} = 5.143$; $p = 0.006$] and cerebral cortex [$F_{(3,13)} = 6.845$; $p = 0.003$] following administration of 4 mg/kg ketamine. We similarly observed an increase in TBARS in the cerebellum, striatum and pre-frontal cortex after an injection of 10 mg/kg ketamine (Fig. 1). These results indicate that lipid peroxidation is stimulated in a rat model of induced schizophrenia.

To investigate oxidative damage to proteins, we evaluated the carbonyl and sulfhydryl content of brain homogenates. Fig. 2 shows an increase in carbonyl content at ketamine doses of 10 mg/kg in the hippocampus [$F_{(3,13)} = 16.764$; $p = 0.001$] and striatum [$F_{(3,15)} = 6.153$; $p = 0.01$] as well as at ketamine doses of 30 mg/kg in hippocampus. We observed a decrease in the prefrontal cortex at 30 mg/kg ketamine. Fig. 3 shows that the sulfhydryl content was decreased at 4 mg/kg ketamine in the hippocampus [$F_{(3,12)} = 55.34$; $p = 0.001$] and cerebral cortex [$F_{(3,8)} = 155.33$; $p = 0.001$], at 10 mg/kg in the cerebellum [$F_{(3,13)} = 242.24$ $p = 0.001$], hippocampus, striatum [$F_{(3,12)} = 3.128$; $p = 0.042$] and cerebral cortex and at 30 mg/kg ketamine in the cerebellum, striatum and cerebral cortex.
Next, we examined the activities of the antioxidant enzymes CAT and SOD in the same cerebral structures. Figs. 4 and 5 show that these activities were altered in all structures tested. Fig. 4 shows that SOD activity was decreased in all doses tested in the pre-frontal cortex \(F(3,10) = 10.34; \ p = 0.005\), cerebellum \(F(3,12) = 2.83; \ p = 0.048\), hippocampus \(F(3,11) = 9.74; \ p = 0.002\) and striatum \(F(3,12) = 6.46; \ p = 0.023\). We also observed an increase in the cerebral cortex at 30 mg/kg ketamine \(F(3,10) = 12.87; \ p = 0.001\). CAT activity (Fig. 5) is decreased in all doses tested in the cerebellum \(F(3,13) = 19.83; \ p = 0.001\) and pre-frontal cortex \(F(3,15) = 4.16; \ p = 0.024\), as well as decreased at 10 mg/kg ketamine in the hippocampus \(F(3,15) = 4.73; \ p = 0.002\) and at 30 mg/kg ketamine in the cerebral cortex \(F(3,12) = 2.08; \ p = 0.034\). We did not observe any alteration in CAT activity in the striatum \(F(3,15) = 1.05; \ p = 0.398\) at these doses.

4. Discussion

Schizophrenia is a complex disease that manifests with a heterogeneous profile across patients. An important clinical distinction involves negative and positive symptoms (Bowie and Harvey, 2006). Recent studies have shown that the blockade of NMDA receptors in adult animals mimics the symptoms of schizophrenia,
and this approach has thus gained popularity as a model in which to study this disease. Supporting this are the findings that antagonizing NMDA receptors in schizophrenic patients exacerbates some psychotic symptoms and has pschotomimetic effects in normal humans (Krystal et al., 1994). In rodents and monkeys, acute sub-anesthetic doses of NMDA antagonists produce a schizophrenia-like symptomatology, including hyperlocomotion, enhanced stereotyped behaviors, cognitive and sensorimotor gating deficits, and impaired social interactions (Lipska and Weinberger, 2000; Pietraszek, 2003; Bressan and Pilowsky, 2003).

In the present study, rats were submitted to different sub-anesthetic doses of ketamine, an NMDA antagonist which induces symptoms similar to those of schizophrenia. We then studied the effect of these different doses of ketamine on parameters of oxidative stress in various regions of the brain. While the pathogenesis of schizophrenia remains unknown, evidence has indicated that there may be a relationship between oxidative stress and the disease (Reddy and Yao, 1996).

Oxidative stress is manifested as an increase in lipid peroxidation end-products, DNA (and often RNA) base oxidation products and oxidative protein damage (Halliwell 2001; Halliwell, 2006). It is an important event that has been related to the pathogenesis of many diseases affecting the central nervous system (CNS), such as neurodegenerative disorders, Parkinson and Alzheimer’s diseases (Fendri et al., 2006), epilepsy, multiple sclerosis, dementia and bipolar disorder (Halliwell, 2001).

The data presented in this study demonstrate that sub-anesthetic doses of ketamine at 4 and 10 mg/kg increased TBARS, indicating an increase in lipid peroxidation. Our data corroborate evidence from human studies showing an elevation in lipid peroxidation products in non-treated schizophrenic patients (Arvindakshan et al., 2003; Mahadik et al., 1998; Petronijevic et al., 2003; Srivastava et al., 2001).

We also demonstrated an increase in the carbonyl content and a decrease in the sulphydryl content of protein, two parameters used to evaluate protein damage, in treated animals when compared to the control group. Protein oxidation is increased in neurodegenerative disorders, advanced age and diseases such as Alzheimer’s, Huntington’s and Parkinson (Buttlerfield and Kanski, 2001). Our results suggest that protein damage is also related to the pathogenesis of schizophrenia. Recent studies showed increased levels of various specific biomarkers of oxidative stress in plasma from schizophrenic patients (Dietrich-Muszalska et al., 2009; Dietrich-Muszalska and Olas, 2009). This group also showed that levels of homocysteine in plasma was higher and that levels of glutathione, cysteine and cysteinylglycine were decreased in patients compared with the control group, suggesting that the amount of carbonyl groups and 3-nitrotyrosine in plasma proteins may be important indicators of protein damage in vivo in schizophrenia (Dietrich-Muszalska et al., 2009; Dietrich-Muszalska and Olas, 2009). Tresadern and Puri (2008) suggested that there is an increase in cerebral mitochondrial oxidative phosphorylational which leads to the formation of superoxide radicals and other reactive oxygen species, in schizophrenic patients with a history of serious and dangerous violent offenses.

Finally, we investigated the effect of sub-anesthetic doses of ketamine on enzymatic antioxidant defenses in the brain of rats by determining SOD and CAT activities. We observed that ketamine decreased the activity of both of these enzymes in almost all structures tested, indicating an effect on another important biomarker of oxidative stress in the brain.

The pathophysiology of schizophrenia is still poorly understood; however the increase in lipid peroxidation, oxidative protein damage and the decrease in enzymatic defenses observed in an animal model of induced schizophrenia indicate that oxidative stress could be related to the development of this disease and these findings may explain, at least in part, the mechanisms which underlie it.

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