ORIGINAL ARTICLE

Effect of isopregnanolone on rapid tolerance to the anxiolytic effect of ethanol

Influência da isopregnenolona na tolerância rápida ao efeito ansiolítico do etanol

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Abstract

Objective: It has been shown that neurosteroids can either block or stimulate the development of chronic and rapid tolerance to the incoordination and hypothermia caused by ethanol consumption. The aim of the present study was to investigate the influence of isopregnanolone on the development of rapid tolerance to the anxiolytic effect of ethanol in mice. Method: Male Swiss mice were pretreated with isopregnanolone (0.05, 0.10 or 0.20 mg/kg) 30 min before administration of ethanol (1.5 g/kg). Twenty-four hours later, all animals we tested using the plus-maze apparatus. The first experiment defined the doses of ethanol that did or did not induce rapid tolerance to the anxiolytic effect of ethanol. In the second, the influence of pretreatment of mice with isopregnanolone (0.05, 0.10 or 0.20 mg/kg) on rapid tolerance to ethanol (1.5 g/kg) was studied. Conclusions: The results show that pretreatment with isopregnanolone interfered with the development of rapid tolerance to the anxiolytic effect of ethanol.

Keywords: Ethanol; Drug tolerance; Anti-anxiety agents; Mice; Alcoholism

Resumo

Objetivo: Estudos prévios têm mostrado que os neuroesteróides podem bloquear ou estimular o desenvolvimento da tolerância rápida e crônica aos efeitos de incoordenação e hipotermia produzidos pelo etanol. O objetivo do presente estudo foi investigar a influência da isopregnenolona sobre o desenvolvimento da tolerância rápida ao efeito ansiolítico do etanol em camundongos. Método: Camundongos suíços, machos, foram pré-tratados com isopregnenolona (0,05, 0,10 ou 0,20 mg/kg) 30 minutos antes da administração de etanol (1,5 g/kg). Após 24 horas, todos os animais foram testados no labirinto em cruz elevado. O primeiro experimento foi realizado com o intuito de selecionar uma dose de etanol que produzisse tolerância rápida ao efeito ansiolítico do etanol. No segundo experimento, o objetivo foi investigar o efeito da isopregnenolona (ISO; 0,05, 0,10 ou 0,20 mg/kg) sobre a tolerância rápida ao etanol (1,5 g/kg). Conclusões: Os resultados mostram que o tratamento prévio com isopregnenolona interferiu no desenvolvimento da tolerância rápida ao efeito ansiolítico etanol.

Descritores: Etanol; Tolerância a drogas; Ansiolíticos; Camundongos; Alcoolismo

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Introduction

Although alcoholism is one of the principal medical problems worldwide, the mechanisms responsible for the various clinical manifestations of this disease remain unclear. Therefore, various studies have been carried out in attempts to gain a better understanding of the mechanisms of action that result in the development of alcohol tolerance and alcohol dependence. It has been suggested that the phenomenon of tolerance to alcohol is one of the factors associated with dependence, and alcohol tolerance has therefore been widely studied. Knowledge of the phenomenon of tolerance to the effects of ethanol has furthered understanding of the implicit nature of the habit of consuming alcoholic beverages, thereby leading to the development of new treatment regimens for this disease.

Tolerance is one of the various criteria that characterize ethanol dependence. It is defined as the need for progressively greater amounts of the substance to reach the state of intoxication or an accentuated reduction of its effect with the constant use of the same quantity of the substance.[1-2]

A temporal approach to the study of tolerance allows us to classify it as acute, rapid or chronic. Acute tolerance is observed during a single episode of ethanol administration, whereas chronic tolerance is generally detected after various days, weeks or months of repeated ethanol administration.[3-4] Rapid tolerance, which is usually detected between 8 and 24 hours after the first ethanol injection,[5-8] has been considered a predictive model of chronic tolerance due to the similarity observed between rats in the two types of tolerance.[9-10]

It has been demonstrated that ethanol affects a variety of neurotransmitter systems, such as those of acetylcholine, monoamine, amino acids and neuroactive steroids.[11-12] Ethanol also exerts significant effects on membranes, ionic channels, second messenger systems and nitric oxide.[13-16] However, some studies suggest that ethanol can stimulate GABAergic neurotransmission and block glutamatergic neurotransmission.[2,17] The effect of ethanol on the GABA-A receptor can contribute to its anxiolytic and sedative effects,[18] resulting in learning impairment and memory loss.[19-20] It has been reported that the development of alcohol tolerance and alcohol dependence seems to be associated with reduced effectiveness of GABA or of the benzodiazepines in some regions of the brain, due in part to the desensitization or down-regulation of the GABA-A receptor.[21-22]

The term "neurosteroid" was proposed by Baulieu in 1981 and has been applied to various steroids that accumulate in the nervous system, independently of the endocrine steroidogenic glands, and can be resynthesized from cholesterol, in both the central and peripheral nervous systems.[23-24] It has been demonstrated that neurosteroids, in addition to their classical genomic action, have nongenomic effects.[25] For example, allopregnanolone, allotetrahydrodeoxycorticosterone, progesterone, epipregnanolone and isopregnanolone (a neuroactive metabolite of progesterone) act as positive modulators,[26-27] whereas pregnenolone sulfate and dehydroepiandrosterone sulfate act as negative modulators of the GABA-A receptor.[28-29] Melchior & Ritzmann demonstrated that the neurosteroids dehydroepiandrosterone sulfate, pregnenolone sulfate and pregnenolone blocked the anxiolytic effect of ethanol in mice.[30-31] In addition, it has been demonstrated that chronic treatment with neurosteroids in mice interferes with tolerance to the antinociceptive effect of morphine and to the effect of benzodiazepines on locomotion.[32-33]

Studies carried out by Barbosa & Morato[6,34-35] revealed that the pretreatment of mice with pregnenolone sulfate or dehydroepiandrosterone sulfate facilitated tolerance to ethanol, whereas epipregnanolone blocked such tolerance. Since the anxiolytic effect of ethanol has been used for several years in the study of the various aspects of ethanol, and since this effect is one of the factors generally recognized as being subjacent to dependence on this drug, the present study investigated the influence of isopregnanolone on the development of rapid tolerance to the anxiolytic effect of ethanol in mice.

Method

1. Animals

This study involved male Swiss mice, aged 3 to 3.5 months, weighing 35-40 g and housed in the animal facility of the Laboratory of Physiology of the Regional University of Blumenau. The mice were maintained in a temperature-controlled environment (23 ± 1°C) on a 12-h light/dark cycle (lights on at 6 am) and were given free access to food and water. The present study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the United States. The experimental protocol was approved by the Ethics Committee for the Use of Animals of the Regional University of Blumenau (process no. CEUA-004/2003).

2. Drugs

The following drugs were used: analytical-grade ethanol (degree of purity = 99.8%; Merck, Darmstadt, Germany) diluted in saline solution (0.9% NaCl) at 14% (p/v) in all experiments; isopregnanolone (3 beta-hydroxy-5 alpha-pregnan-20-one; Research Biochemicals International, Natick, MA, USA), prepared in the proper concentrations in saline solution; the saline solution was prepared with NaCl (Merck) in distilled water at a concentration of 0.9%. All of the solutions were administrated intraperitoneally, and all were injected in volumes of 1 ml/kg of body weight, except for the ethanol, the proportion of which was adjusted according to the dose utilized.

3. Elevated plus-maze test

The elevated plus-maze (EPM) is a T-shaped wooden maze positioned at 56 cm above the floor and consists of two open arms (30 x 5 cm) and two closed arms (30 x 5 x 30 cm), directly opposite each other, with a 0.5-cm high acrylic protector surrounding the open arms in order to prevent the animals from falling. The apparatus was maintained in a partially dark room, illuminated by red light (44 lux). Prior to the EPM test, the animals were placed in a wooden arena (40 x 40 cm) for five minutes to become accustomed to the test environment. The animals were placed in the EPM for five minutes in order to record the behavioral measurements, which consist of determining the frequency of entry into the open arms (FEO) and into the closed arms (FEC), as well as the elapsed time spent in the open arms (TSO) and in the closed arms (TSC). The percentage of FEO (%FEO) and of TSO (%TSO) were calculated according to the formulas:

$$\%FEO = \frac{FEO \times 100}{FEO + FEC} \qquad \%TSO = \frac{TSO \times 100}{TSO + TSC}$$

The EPM model is based on, among other factors, the natural aversion rodents show to the open arms of the labyrinth since, when rats are forced to stay in the open arms, they present behavioral and physiological manifestations of fear, such as freezing, defecation and increased levels of plasma corticosteroids. Consequently, they spend more time in the

closed arms. The proportion of the total exploration of the open arms is a measure of anxiety. Therefore, an increase in the percentage of entries and in the time spent in these arms is considered indicative of the anxiolytic action of drugs.

4. Experimental procedure

Experiment 1 – Effect of different doses of ethanol on the development of rapid tolerance to the anxiolytic effect. This experiment was carried out with the objective of selecting a dose of ethanol that would result in rapid tolerance to the anxiolytic effect of this drug. On day 1, 30 mice (three groups of 10) received ethanol (1.5 g/kg), and 30 others (also in three groups of 10) received saline. The animals were then returned to their home cages. On day 2, all of the animals received ethanol (0.75, 1.0 or 1.5 g/kg), and the anxiolytic response was measured after 10 minutes to evaluate the degree of rapid tolerance.

Experiment 2 – Effect of isopregnanolone on the rapid tolerance to the anxiolytic effect of ethanol. On day 1 of the experiment, 60 mice (three groups of 20) were pretreated with saline, and another 60 (also in three groups of 20) were pretreated with isopregnanolone (0.05, 0.10 or 0.20 mg/kg, respectively). After 30 minutes, each group was divided into two subgroups of 10 mice each, and the mice in one of each pair of subgroups received 1.5 g/kg of ethanol, whereas those in the corresponding subgroups received 1.5 g/kg of saline. The animals were then returned to their home cages. On day 2, all of the animals were treated with a dose of 1.5 g/kg of ethanol and the anxiolytic response was evaluated. The 1.5 g/kg dose of ethanol was chosen because it produced more evident tolerance (Figure 1).

5. Statistical analysis

The results were evaluated using Student's t-test or one-way ANOVA. The post hoc test applied was the Tukey test. Values of $p = 0.05$ were considered statistically significant, and the results are presented as mean ± SEM.

Results

Figure 1 illustrates the effect of different doses of ethanol on the development of rapid tolerance to the anxiolytic effect of ethanol. Administration of a 1.5 g/kg dose of ethanol on day 2 resulted in a reduction in the anxiolytic effect. Ethanol administration decreased the %FEO [$t_{(1.18)} = 3.825$; $p < 0.0024$] and the %TSO [$t_{(1.18)} = 3.692$; $p < 0.0027$].

The results of experiment 2 are presented in Figure 2. On day 2, pretreatment with a 0.20 mg/kg dose of isopregnanolone blocked the development of rapid tolerance to the anxiolytic effect of ethanol. However, there was a significant decrease in the anxiolytic effect in the control groups that received ethanol on both days (EE), suggesting that the 1.5 g/kg dose of ethanol results in rapid tolerance. In the ANOVA, the factors that were found to have a significant effect were as follows: ethanol treatment (%FEO [$F_{(1.36)} = 27.75$; $p < 0.0001$] and %TSO [$F_{(1.36)} = 16.43$; $p < 0.0003$] – %FEO [$F_{(1.36)} = 22.65$; $p < 0.0001$] and %TSO [$F_{(1.36)} = 12.76$; $p < 0.0010$] – %TSO [$F_{(1.36)} = 6.56$; $p < 0.014$] (Figures 2A, 2B and 2C); and pretreatment with isopregnanolone (%FEO [$F_{(1.36)} = 12.99$; $p < 0.0009$] and %TSO [$F_{(1.36)} = 15.09$; $p < 0.0004$]) (Figure 2C). The post hoc analysis (Tukey test) confirmed that the 0.20 mg/kg pretreatment with isopregnanolone blocked the rapid tolerance compared to the control groups, although the 0.10 mg/kg dose only partially blocked the development of such tolerance (Figure 2B). However, the 0.05 mg/kg dose had no effect on the development of rapid tolerance to ethanol (Figure 2A).

Discussion

The results of the present study clearly demonstrate the development of rapid tolerance to the anxiolytic effect of ethanol in mice receiving a 1.5 g/kg dose on two consecutive days. These data corroborate the results obtained in other experiments, in which rapid tolerance to the incoordination and hypothermia caused by ethanol consumption was achieved at certain dose levels.[6-7,35-38] In addition, studies carried out by Khanna et al. revealed that rapid tolerance occurs when ethanol is administered on both test days (intoxicated practice) and also in the dummy test (measurements were not actually recorded until the second day of the protocol), suggesting that the behavioral experience on an inclined plane or on the hypothermia test is not crucial for the development of rapid tolerance to ethanol.[36] Our results are also in accordance with those of that study since our animals were tested in the elevated plus-maze only on the second day. Moreover, the results of studies carried out by Bertoglio & Carobrez suggest that previous experience in the elevated plus-maze affects the anxiolytic effect of ethanol and of phenobarbital, a phenomenon known as "one-trial tolerance".[39]

In the present study, the acute administration of isopregnanolone, a mild positive modulator of the GABA-A

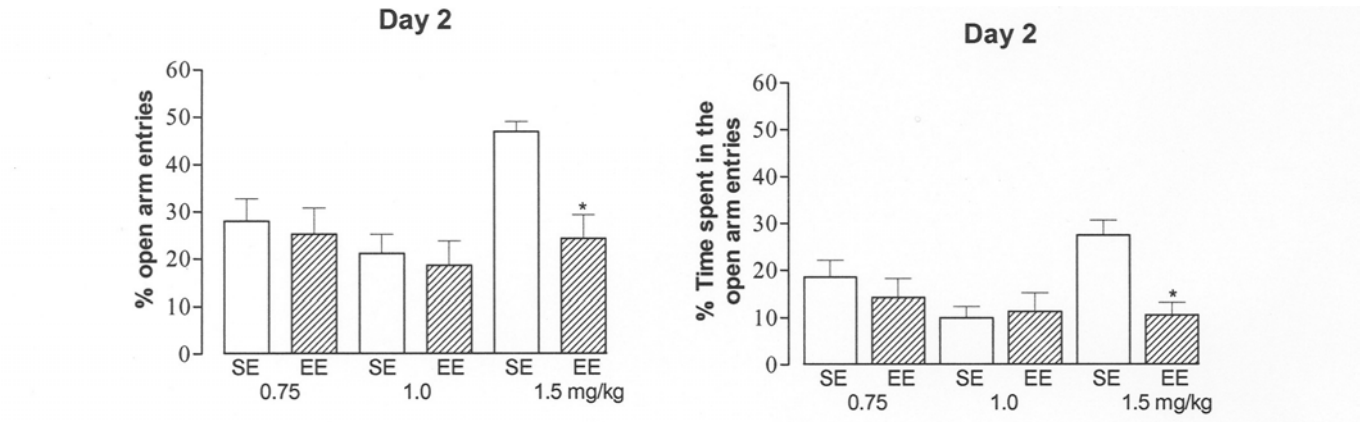

Figure 1 – Development of rapid tolerance to the anxiolytic effect induced by different doses of ethanol in mice submitted to the elevated plus-maze test. On day 1, three groups received saline (S), and another three groups received intraperitoneal (i.p.) injections of ethanol (E; 0.75; 1.0 or 1.5 g/kg) but were not tested. Rapid tolerance was observed on day 2, when all mice were treated with ethanol (0.75; 1.0 or 1.5 g/kg, i.p.) and tested 10 minutes after the injections. The results represent the mean ± SEM of the percentage of entries into open arms and time spent in the open arms of 10 animals per group (see the Methods section for details). *p 0.05 compared to respective control group (Student's *t*-test).

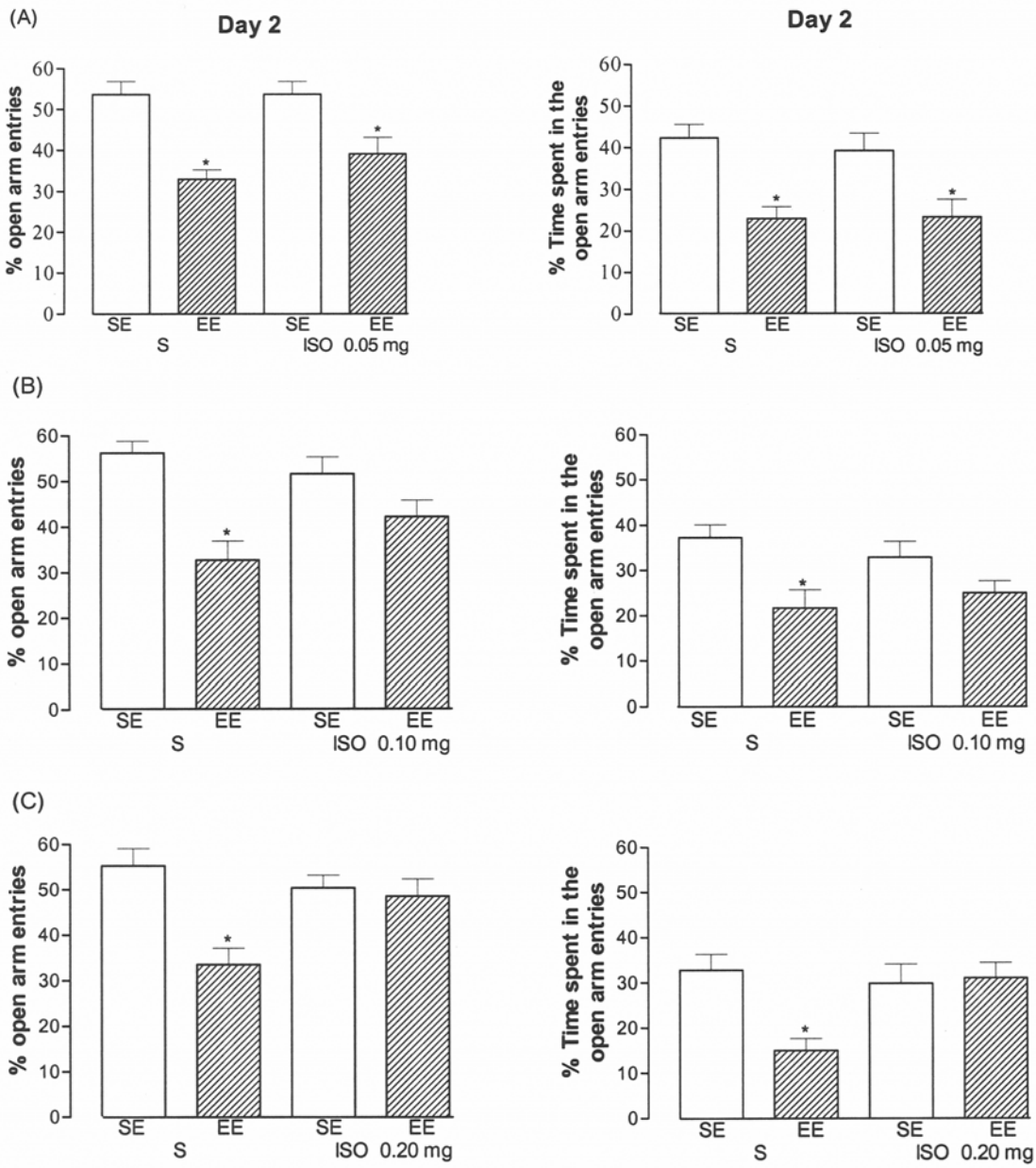

Figure 2 – Effect of isopregnanolone on the development of rapid tolerance to the anxiolytic effect of ethanol in mice. Six groups received saline (S), and another six groups received intraperitoneal (i.p.) injections of isopregnanolone (ISO) in the doses of 0.05 (A), 0.10 (B) or 0.20 (c) mg/kg 30 minutes before the administration of saline (S) or ethanol (E; 1.5 g/kg i.p.) on day 1 but were not tested. Rapid tolerance to the effects of ethanol was observed on day 2, when all of the animals were treated with ethanol (EE) at the dose of 1.5 g/kg, i.p. The results represent the mean ± SEM of the percentage of entry into the open arms and time spent in the open arms of 10 animals per group (see the Methods section for details). *p 0.05 compared to the respective control group (ANOVA + Tukey).

receptor,[27] prior to the administration of ethanol significantly blocked the development of rapid tolerance to the anxiolytic effect of the ethanol. These results are consistent with those of previous studies on the influence of neurosteroids on rapid and chronic tolerance to the incoordination and hypothermia induced by ethanol consumption in mice.[6,34-35]

The doses of isopregnanolone used in the present study did not have any residual effect on the performance of the animals on the second day. In addition, the effect of this neurosteroid seems to be pharmacodynamic rather than pharmacokinetic since our results and those of previous studies have shown that the acute administration of neurosteroids does not interfere with the pharmacokinetics of ethanol in mice or rats.[6,35,40] However, Barbaccia et al. reported that a low dose of ethanol (1 g/kg) increased levels of allopregnanolone and allotetrahydrodeoxycorticosterone in the cerebral cortices and hippocampi in a lineage of rats that prefer alcohol, when compared to those presented by a lineage of rats that do not prefer alcohol.[41] O'Dell et al. also showed that the administration of ethanol (2 g/kg) increased the levels of these neurosteroids in the frontal cortices of rats.[42] Furthermore, the findings of other studies have suggested that acute administration of ethanol decreases the levels of dehydroepiandrosterone sulfate in the brains of rats.[43]

Drugs that act on the GABA-A receptor system can influence tolerance to ethanol. It has been demonstrated that muscimol

blocks the development of tolerance to ethanol-induced incoordination,[6] whereas agonists and antagonists of GABA-B receptor have been shown to block and stimulate tolerance, respectively.[37] Recent experiments have demonstrated that neurosteroids exert positive and negative modulatory influence on the GABA-A and NMDA receptors, as well as blocking or stimulating the development of a tolerance to ethanol.[6,34-35] These findings suggest that tolerance to ethanol is related to the GABA-A system, the NMDA system or both.

It is known that neurosteroids interact with ethanol. The positive modulators of GABA-A receptor (allopregnanolone and pregnenolone) increased the sleep time and motor loss induced by ethanol in mice.[44-45] Therefore, ethanol and some neurosteroids might have similar effects associated with the GABA-A receptor complex, which may explain the interactions between these drugs. The chronic administration of ethanol has been correlated with a decrease in GABA-A receptor-mediated response sensitivity in the central nervous system.[46-48] It has also been demonstrated that the chronic administration of allopregnanolone, a positive modulator of the GABA-A receptor, down-regulates the GABA-A receptor.[49] In addition, it has been shown that removing neurosteroid precursors through oophorectomy does not directly affect the neuro-adaptation of the GABA-A receptors associated with chronic exposure to ethanol.[50] Based on such evidence, one would think that the isopregnanolone treatment administered on day 1 would have increased the ethanol-induced down-regulation of the GABA-A receptor system, thereby increasing tolerance to ethanol on day 2. However, the opposite was observed; the ethanol-induced tolerance was blocked isopregnanolone administration. Therefore, it seems that the influence of this neurosteroid on the development of tolerance to ethanol seems to be unrelated to alterations in the down-regulation of the GABA-A receptors. In view of this, other mechanisms may be involved in the development of rapid tolerance to ethanol.

Taken as a whole, our results suggest that isopregnanolone influences the development of rapid tolerance to ethanol, possibly through a mechanism that involves the GABA-A receptor complex.

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