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BEHAVIORAL AND BIOCHEMICAL CHANGES AFTER MULTIPLE ADMINISTRATION OF ETHANOL ALONE AND IN COMBINATION WITH NIFEDIPINE

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Summary. The effect of the dihydropyridine calcium channel blocker nifedipine on ethanol withdrawal symptoms and the activity of neuronal nitric oxide synthase (nNOS) was investigated in rats made dependent on ethanol by repeated ethanol administration for eight consecutive days. Forty eight hours after the last administration, behavioral changes were evaluated and the withdrawal symptoms were observed. Co-administration of nifedipine attenuates these symptoms, which correlate with the biochemical assay of nNOS. The data on the nNOS activity showed that ethanol, administered alone, significantly increased by 74% nNOS activity. Nifedipine itself did not change the enzyme activity, while in the combination group nifedipine decreased the brain nNOS activity, induced by multiple ethanol administration. The results of our study suggest that nifedipine may play an important role in reduction the severity of the withdrawal symptoms and related to it changes in nNOS.

Key words: ethanol, withdrawal, nifedipine, nNOS

ПОВЕДЕНЧЕСКИ И БИОХИМИЧНИ ПРОМЕНИ СЛЕД МНОГОКРАТНО ПРИЛАГАНЕ НА ЕТАНОЛ, САМОСТОЯТЕЛНО И В КОМБИНАЦИЯ С НИФЕДИПИН

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Резюме. Проследен е ефектът на дихидропиридиновия калциев блокер нифедипин върху симптомите на етанолна абстиненция и върху активността на невроналната азотоксидсинтаза (nNOS) при плъхове, развили етанолна зависимост в резултат на многократно (8-дневно) прилагане на алкохол. Поведенческите ефекти са оценени на 48-ия час след последното прилагане на етанол, като се отчитат симптоми на абстиненция. Едновременното прилагане с нифедипин смекчава симптомите на отнемане, което корелира и с биохимичното определяне на nNOS. От резултатите се установява, че самостоятелното прилагане на етанол води до значимо повишаване, със 74% на активността на nNOS. Нифедипин, приложен самостоятелно, не променя активността на ензима. В комбинираната група води до намаляване на повишената от многократното прилагане на етанол активност на nNOS. Резултатите от нашето проучване предполагат, че нифедипин може би играе важна роля в намаляване тежестта на етанолна абстиненция чрез повлияване активността на nNOS.

Ключови думи: етанол, абстиненция, нифедипин, nNOS

Introduction

Chronic and excessive alcohol abuse is marked by a number of biochemical and physiological changes in the central nervous system (CNS). Ethanol addiction, developed after multiple ethanol administration, is a complex phenomenon that includes tolerance, craving, physical dependence and withdrawal syndrome. It has been recognized for some time that N-methyl-D-aspartate (NMDA) nitric oxide (NO) cascade may play a role in voluntary ethanol consumption [9] and in neurobiological alterations induced by chronic ethanol consumption during the development of ethanol tolerance, dependence, and withdrawal [1]. In one of their studies, Uzbay et al. [12] showed that the nNOS inhibition by L-NAME and 7-NI alleviates

the signs of ethanol withdrawal. The activation of NMDA/NO cascade involves an increase of Ca^{2+} into the neurons and consequent activation of Ca-dependent nNOS [11]. In relation to this information, there are literature data that calcium channel blockers of 1,4-dihydropyridine type, such as nifedipine, might influence the processes of tolerance and withdrawal, due to changes in Ca^{2+} homeostasis. In our previous studies, we demonstrated that nifedipine, co-administered with morphine, attenuates the symptoms of opiate withdrawal which correlated with the changes in neuronal nitric oxide synthase (nNOS) [14].

The objective of our study was to investigate the influence of nifedipine on behavioral and biochemical changes after multiple administration of ethanol in rats.

Material and Methods

Animals

Male Wistar rats (weighing 200 g to 230 g) were housed in Plexiglas cages (3 per cage) under standard laboratory conditions: at 20 ± 2°C and 12 h alternating light/dark cycles. Food and tap water were provided *ad libitum*. The animals were purchased from the National Breeding Centre, Slivnitza, Bulgaria. All experiments were performed after at least one week of adaptation to this environment. All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) [6] were strictly followed throughout the experiment.

Experimental design

The animals were divided into four groups (n = 6). The first group was treated with nifedipine at a dose of 5 mg kg^{-1} i.p., [2] twice daily for 8 days. The second group was treated with 40% ethanol (2.9 g kg^{-1}) p.o. by gavages twice daily for 8 days. The third group was receiving nifedipine (5 mg kg^{-1} i.p.) and 30 min after that 40% ethanol (2.9 g kg^{-1} p.o.). This combination was also administered twice daily for 8 days. The fourth group included control animals, treated with saline, was involved in the experiment from the very beginning and housed under the same standard laboratory conditions as the treated animals in groups 1-3. Forty eight hours after the last administration the observation test for ethanol withdrawal was performed according to the method of Becker et al. [4]. Than the animals were sacrificed and nNOS activity was assessed.

Chemicals and reagents

All the reagents used were of analytical grade. The drugs used in this study, ethanol and nifedipine hydrochloride, as well as other chemicals, sucrose, Tris, DL-dithiotreitol, phenylmethylsulfonyl fluoride, potassium phosphate, calcium chloratum ($CaCl_2$), magnesium chloratum ($MgCl_2$), L-arginine, L-valine, hemoglobin bovine, beta-Nicotinamide adenine dinucleotide 2`-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (fraction V) were purchased from Sigma Chemical Co (Germany).

Test for physical dependence in rats

Physical dependence capacity was recorded 48 hours after the last ethanol administration, alone and in combination with nifedipine, using the Observation behavioral test, described by Becker et al. [4].

Preparation of brain tissue extracts and assessment of nNOS activity

Rats were decapitated and the brains were minced and homogenized in 10 volumes of buffer, containing 320 mmol L^{-1} sucrose, 50 mmol L^{-1} Tris, 1 mmol L^{-1} DL-dithiotreitol, 100 μg/L phenylmethylsulfonyl fluoride (pH = 7.2) according to the method used by Knowels and Moncada [8]. The homogenates were then centrifugated at 17 000 x g for 60 min. The protein content was measured by the method of Lowry [10] with bovine serum albumin as a standard.

nNOS activity was measured spectrophotometrically using the oxidation of oxyhemoglobin to methemoglobin by NO (18) with slight modifications. The incubation medium contained 40 mM potassium phosphate buffer, pH = 7.2, 200 μmol L^{-1} $CaCl_2$, 1 mmol L^{-1} MgCl2, 100 μmol L^{-1} L-arginine, 50 mmol L^{-1} L-valine, 2.6 μmol L^{-1} oxyhemoglobin, 100 μmol L^{-1} NADPH and brain extract. The change in the difference in absorbance at 401 nm and 421 nm was monitored with a double split beam spectrophotometer (Spectro UV-VIS Split), at 37°C. The activity of the enzyme was expressed in nmol/min/mg, using the milimolar extinction coefficient of methemoglobin 77.2 $M^{-1}cm^{-1}$.

Statistical analysis

Analyses were performed using EXCEL for Windows computer program. Statistical analysis included ANOVA and the Student's t-test. Probability values less than 0.05 were considered significant. The results were presented as mean values (± SD) of 6 animals per group. For each of the examined parameters three paralel samples were used.

Results

The results of the observation test of the withdrawal symptoms are shown in table 1. Forty eight hours after the last ethanol administration the animals showed all the withdrawal symptoms, while in the nifedipne + ethanol group the animals did not exert symptoms to the same extent. The animals in the control and nifedipine treated groups did not show withdrawal symptoms.

The effect of ethanol and nifedipine on nNOS activity in rat brain is shown in Table 2. The results demonstrate that ethanol per se, increased the activity of nNOS by 74 % ($p < 0.05$). Nifedipine alone did not significantly change the activity of brain NOS, compared to the levels of control group, while in combination with ethanol, nifedipine significantly decreased, by 28% ($p < 0.05$), the enzyme activity, enhanced by multiple ethanol administration.

Discussion

In the present study, the influence of nifedipine on behavioral and biochemical changes, caused by multiple ethanol administration, was investigated. Ethanol is a powerful addictive drug of abuse, causing tolerance and dependence development and withdrawal syndrome. One of the neurobiological mechanisms underlying these processes are thought to be an activation of NMDA/NO cascade and consequent increase of Ca^{2+} influx into the neurons [7]. The increased Ca^{2+} concentration, on the other hand, results in an increase of nNOS activity and excessive production of NO that plays an important role both as neurotransmitter and neurotoxicant [3]. These changes in the brain are considered responsible for the behavioral effects, caused by abrupt discontinuation of ethanol after repeated administration. In the present study, 48 hours after the last ethanol application, the animals showed withdrawal symptoms – tremor, piloerection, open space avoidance, unusual body position, anxiety. On the biochemical level, this corresponds with significantly increased nNOS activity. Vetulani [13] outlined that blockers of L-type Ca^{2+} channels, like 1,4-dihydropiridines are interesting for their potential application as anti-addiction agents. They appear to play a principal role in the regulation of adaptive changes in the central nervous system by maintaining different types of drug dependence and withdrawal, mainly by influencing the Ca^{2+} homeostasis. Our results showed that nifedipine, administered along with ethanol, attenuated the ethanol withdrawal, which is probably due to the detected decrease in nNOS activity, enhanced by multiple cocaine administration (table 2).

Table 1. *Observation test for assessment of ethanol withdrawal symptoms*

Symptoms in rats	Control group	Nifedipine	Ethanol	Nifedipine + ethanol	Corresponding symptoms in humans
Reduced intake of food and water	–	–	+++	–	Gastro-intestinal disturbances (nausea and vomiting)
Tremor	–	–	+++	–	Tremor
Piloerection	–	–	++	–	Sweating
Open space avoidance	–	–	++	–	Anxiety
Unusual body position	–	–	+++	+	Autonomic hyperactivity
Anxiety	–	–	+++	–	Anxiety

+++ high, ++ medium , + low

Table 2. *nNOS activity in rat brain after multiple administration of 40 % ethanol (2.9 mg kg^{-1}) and nifedipine (5 mg kg^{-1}), alone and in combination*

Group	nNOS activity (nmol/min/mg)	Effect vs conrol (%)	Effect vs ethanol (%)
Control	0.604 ± 0.04	100	
Nifedipine	0.597 ± 0.08	98.9	
Ethanol	1.050 ± 0.016*	174	100
Nifedipine + Ethanol	0.755 ± 0.10+	125	72

Note: Data are expressed as mean ± SD of six animals

*$p < 0.05$ vs control group; +$p < 0.05$ vs ethanol treated group

These data support the behavioral study carried out by Colombo et al. [5] that investigated the effect of 1,4-dihydropiridine darodipine on ethanol withdrawal in rats. The authors proved that six days co-administration of darodipine prevented the severity of ethanol withdrawal signs and that the presence of darodipine in the central nervous system during the adaptative responses to ethanol is necessary to reduce the severity of the withdrawal syndrome.

On the basis of the experiments carried out and the results obtained, we could suggest that the 1,4-dihydropiridine calcium channel blocker nifedipine may be useful for maintaining the ethanol tolerance and dependence development, as well as for attenuating the ethanol withdrawal syndrome.

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