DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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INFLUENCE OF NITRIC OXIDE SYNTASE INHIBITORS ON THE EFFECTS OF ETHANOL AFTER ACUTE AND CHRONIC ETHANOL ADMINISTRATION AND WITHDRAWAL

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ABBREVIATIONS

ANOVA	analysis of variance
BH_4	(6*R*)-5,6,7,8-tetrahydro-L-biopterin
CAPON	carboxyl-terminal PDZ ligand of NOS
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
EDRF	endothelium derived relaxing factor
FAD	flavin adenine dinucleotide
FMN	flavin adenine mononucleotide
GABA	gammaaminobutyric acid
NADPH	nicotine adenine dinucleotide phosphate
L-NAME	N^G-nitro-L-arginine methyl ester
L-NOARG	N^G-nitro-L-arginine
7-NI	7-nitroindazole
NMDA	N-methyl-D-aspartate
NO	nitric oxide
eNOS	endothelial nitric oxide synthase
iNOS	immunological nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
PIN	protein inhibitor of nNOS

INTRODUCTION

Ethanol is the second most widely used psychotropic drug in the world after caffeine. Ethanol is also most widely abused drug with the greatest cost to society. It has been estimated that 20–40% of patients in large urban hospitals are hospitalized because of illnesses caused or worsened by ethanol abuse (Harper *et al.* 2003).

Of 741 autopsies carried out by Tartu Bureau of Forensic Medicine in 2003 acute poisoning with ethanol was the cause of death in 46 (6,2%) and long-term alcohol abuse together with pathological changes in organism in 43 cases (5,8%).

Understanding ethanol's mechanism of action would provide a scientific basis for the treatment of alcoholism.

In spite of extensive studies ethanol's exact mechanism of action remains unknown. It has been demonstrated that ethanol has impact on several neurotransmitter systems — *e.g.* GABAergic, dopaminergic, serotonergic, *etc.*

It has been proposed that some of the effects of ethanol are mediated through nitric oxide (NO), a gaseous mediator that is synthesized by enzyme nitric oxide synthase (NOS) from L-arginine (for reviews see Adams and Cicero 1998; Lancaster 1992). It has been demonstrated that NOS inhibitors, NO donors and NO precursor L-arginine influence ethanol intoxication and withdrawal. NOS inhibitors have also been proposed as possible treatments of ethanol-induced excitotoxicity and ethanol dependence (Lancaster 1995).

The aim of our work was to further study the interaction of ethanol and L-arginine — NOS — NO pathways. For this purpose we observed the effects of NOS inhibitors 7-nitroindazole (7-NI), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NOARG) on the effects of ethanol after acute and chronic ethanol administration and withdrawal.

1. REVIEW OF LITERATURE

1.1. ETHANOL'S MECHANISM OF ACTION

Since the classic works of Meyer and Overton (Meyer 1901; Overton 1896) it was presumed for almost a hundred years that ethanol exerts its effects by dissolving in lipid membranes and thereby altering the function of embedded receptors and ion channels. However, it has been demonstrated that ethanol alters the properties of membranes only at concentrations much higher (>100 mM) (Fadda and Rossetti 1998) than those obtained *in vivo* (5–50 mM) and having effect on the function of ion channels and receptors (Lovinger *et al.* 1989; White *et al.* 1990). Therefore, during the last 10–15 years it has been proposed that ethanol acts directly on membrane proteins — *i.e.* receptors and ion channels — producing conformational changes that alter their function (Harris 1999; Lovinger 1997).

A direct action on proteins is suggested by the potency cutoff effect, the abrupt decline or plateau in the biological potency of alcohols as their molecular size is increased beyond a certain value (Davies 2003; Zuo *et al.* 2001). For example, once the length of alcohol's backbone exceeds 12 carbons, there is no longer an effect on $GABA_A$ receptor — chloride ionophore complex (Davies 2003). Different cutoff values for different receptors suggest interaction of alcohols with rigid conformational pockets, having different size on each receptor (Peoples *et al.* 1996). Ethanol binding sites have been described on N_M-acetylcholine (Forman and Zhou 1999) N_N-acetylcholine (Yu *et al.* 1996), 5-HT_3 (Zhang *et al.* 2002), $GABA_A$, glycine (Mihic *et al.* 1997) and NMDA receptors (Wright *et al.* 1996). It has been proposed that ethanol has effect on virtually every neurotransmitter system (Dodd *et al.* 2000). The complexity of ethanol's effects relies on its chemical structure, characterized by the absence of asymmetric carbon, excluding stereoselectivity, and by the presence of hydrophilic hydroxyl group and lipophilic aliphatic moiety at the opposite ends of the molecule (Fadda and Rossetti 1998). Through the hydroxyl group ethanol forms hydrogen bonds with proteins and phospholipids in cell membrane (Barry and Gawrish 1994), alters the solvation of ligands and ions interacting with receptors (Yurttas *et al.* 1992) and through the aliphatic moiety interacts with nonpolar domains of macromolecules (Fadda and Rossetti 1998).

1.2. NITRIC OXIDE

1.2.1. Discovery of NO's function as neurotransmitter

In 1980 Furchgott and Zawadzki reported that acetylcholine induced vasodilatation only in the presence of intact endothelium (Furchgott and Zawadzki

1980). Further studies revealed the role of reactive mediator EDRF (endothelium derived relaxing factor) (Furchgott *et al.* 1984), later shown to be nitric oxide (NO).

In contrast to "classic" neurotransmitters — *e.g.* acetylcholine and noradrenaline — NO is neither stored in synapse nor released by exocytosis but is synthesized on demand by enzyme NO synthase (NOS) and simply diffuses from nerve terminals (Esplugues 2002).

1.2.2. NOS subtypes, regulation of NOS activity, NO targets and inactivation

NOS is a homodimeric cytochrome P_{450} monooxygenase analog (Bryk and Wolff 1999). Biochemistry and cloning have enabled to identify three separate NOS genes and corresponding enzymes, named either by the tissue or the order in which they were cloned (Yun *et al.* 1996). Neuronal NOS (nNOS, Type I NOS) was cloned from cerebellum (Bredt *et al.* 1991), immunological NOS (iNOS, Type II NOS) was cloned from macrophages (Xie *et al.* 1992) and endothelial NOS (eNOS, Type III NOS) was cloned from endothelial cell culture (Lamas *et al.* 1992). nNOS has several variants with distinct cellular and tissue localization — nNOSα, nNOSβ, nNOSγ, nNOSμ and nNOS-2 (Esplugues 2002). For example, nNOSμ is specifically localized in the skeletal muscle (Bredt 2003; Stamler and Meissner 2001). All NOS isoforms share similar general structure and consist of a single polypeptide chain containing oxygenase and reductase domains and binding sites to calmodulin and electron donors (Bryk and Wolff 1999; Mungrue *et al.* 2003). In contrast to other redox enzymes, that usually employ a single electron donor, nNOS utilizes nicotinamide, NADPH, FMN, FAD, BH_4 and heme (Boehning and Snyder 2003).

nNOS activity in the central nervous system (CNS) is mainly regulated by intracellular calcium. An increase in calcium levels, induced by action potential or activation of NMDA receptors, causes calmodulin binding to nNOS and its activation. Inactivation of nNOS is caused by a decrease in calcium levels and calmodulin dissociation, by phosphorylation through protein kinases and by endogenous nNOS inhibitors — protein inhibitor of nNOS (PIN) and carboxyl-terminal PDZ ligand of NOS (CAPON) (Esplugues 2002; Stamler and Meissner 2001).

iNOS is induced by bacterial lipopolysaccharide and cytokines (Sethi and Dikshit 2000). An older nomenclature classifies NOS into the constitutive (cNOS) and inducible isoforms (iNOS). According to this classification cNOS includes neuronal and endothelial NOS and is regulated by intracellular calcium and inducible isoform (iNOS) includes immunological NOS. However, this classification is considered unreliable because, in addition to calcium levels and cytokines, all isoforms are regulated dynamically by numerous other factors like

tissue injury, age, drugs, hormones, hypoxia, stress, physical exercise and fatigue, *etc.* (Esplugues 2002; Stamler and Meisner 2001).

Most of NOs physiological actions are mediated by binding to enzymes and proteins and altering their function (Pagliaro 2003). The main routes for the action of NO are the generation of cyclic guanosine monophosphate (cGMP) and selective and reversible *S*-nitrosylation of different proteins (Ahern *et al.* 2002; Hess *et al.* 2001).

By stimulating guanylyl cyclase (sGC) and the formation of cGMP NO relaxes blood vessels (DeRubertis and Craven 1976). Approximately a hundred proteins have been identified as substrates for *S*-nitrosylation (Hess *et al.* 2001). Through *S*-nitrosylation NO activates or inhibits different ion channels and receptors. For example, *S*-nitrosylation activates L-type Ca^{2+} channels (Poteser *et al.* 2001) and inhibits NMDA receptors (Choi *et al.* 2000).

Furthermore, NO has been shown to influence the activity of 63 genes, regulating neuronal development, DNA replication, protein metabolism and anti-apoptotic proteins (Li *et al.* 2004).

NO is rapidly inactivated, having a very short half-life (3–6 s) (Gerlach *et al.* 2001; Palmer *et al.* 1988). Mechanism of inactivation is NOs antoher difference from "classic" neurotransmitters. While the activity of "classic" neurotransmitters is terminated either by re-uptake or enzymatic degradation, the inactivation of NO follows its reaction with substrate (Esplugues 2002). Unreacted NO has been assumed to simply diffuse away from target areas and decay spontaneously into nitrites and nitrates (Lowenstein *et al.* 1994), but recent studies have shown enzymatic degradation by NO oxidase (Bredt 1999; Eiserich *et al.* 2002).

1.2.3. Functions of NO and NOS in organism

NOS subtypes are widely distributed in the organism — *e.g.* cytokines can induce iNOS in all somatic cells (Sethi and Dikshit 2000). The localization and function of NOS subtypes is not limited to the tissue they are named after. Thus, iNOS has effect on lipolysis (Andersson *et al.* 1999) and vasodilatator responses (Briones *et al.* 1999) and eNOS is involved in memory processes (Frisch *et al.* 2000).

nNOS has diverse functions in the central and peripheral nervous system (for review see Dawson and Dawson 1994; Esplugues 2002). nNOS is involved in the regulation of responses to pain and stress, neuronal damage and neuroprotection, food and water intake, aggressive behavior, sleep and circadian rhythms (Bilbo *et al.* 2003, Calapai *et al.* 1998a, 1998b; Chiavegatto and Nelson 2003; Esplugues 2002; Kriegsfeld *et al.* 1999; Monti *et al.* 1999).

iNOS mediates the cytotoxic and cytostatic effect of NO against pathogens and tumor cells (Tuynman *et al.* 2003). In response to lipopolysaccharides macrophages generate large amounts of NO sufficient to kill bacteria or tumour cells (Snyder and Ferris 2000).

eNOS is involved in the regulation of vascular tone (for review see Ignarro 2002), including penile erection (Burnett *et al.* 1998) and ejaculation (Kriegsfeld *et al.* 1999) and in reproductive function in females (McCann *et al.* 1999). Subtypes of NOS have also multiple effects on hormone secretion and reproductive function (for review see Dixit and Parvizi 2001).

NO has numerous effects on cell damage through several mechanisms (for review see Dröge 2001; Kendall *et al.* 2001; Stewart and Heales 2003). Without an adequate delivery of L-arginine and co-factors, instead of NO production, NOS transfers free electrons to oxygen and produces free oxygen radicals (Schulz *et al.* 2004). NO can also react with biomolecules, forming cytotoxic compounds (Kendall *et al.* 2001) and with oxygen molecules, forming reactive oxygen species (Kim *et al.* 2001).

1.2.4. NOS inhibitors and their effects

During the last decades different NOS inhibitors with different potency and selectivity towards NOS subtypes have been synthesized. Older NOS inhibitors N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME) inhibit both nNOS and eNOS, resulting in vasoconstriction and hypertension at higher doses and during chronic administration (Wang *et al.* 1995). 7-nitroindazole (7-NI), a selective nNOS inhibitor, does not inhibit eNOS *in vivo* and does not increase blood pressure (Moore *et al.* 1993). In addition to their effect on NOS L-NOARG, L-NAME and 7-NI have effect on the function of serotonergic and dopaminergic systems (for review see Kiss 2000; Prast and Philippu 2001). For example, 7-NI increases and L-NAME decreases the release of dopamine in the brain (Kiss *et al.* 1999). It has been proposed that NOS inhibitors elicit their antidepressant-like effect in the forced swimming test through a serotonin dependent mechanism (Harkin *et al.* 2003).

The effects of NO donors, NO precursor L-arginine and NOS inhibitors on the behaviour of laboratory animals have been extensively studied. While 7-NI induces an anxiolytic effect in the plus-maze test (Dunn *et al.* 1998; Volke *et al.* 1997; Yildiz *et al.* 2000) with L-NOARG and L-NAME both anxiolytic (Czech *et al.* 2003; Faria *et al.* 1997; Guimarães *et al.* 1994; Volke *et al.* 1995) or anxiogenic (De Oliveira *et al.* 1997; Monzón *et al.* 2001; Vale *et al.* 1998) effects have been reported. Surprisingly, despite their anxiolytic effect, NOS inhibitors antagonize the effects of benzodiazepine anxiolytic chlordiazepoxide on food intake (Czech 1996) and the anxiolytic effects of chlordiazepoxide and nitrous oxide in the plus-maze test (Quock and Nguyen 1992; Caton *et al.* 1994) and in the light/dark exploration test (Li and Quock 2001).

Controversial results can be explained with differences in doses used, animal species, routes of administration, different behavioural models and possible other factors — *e.g.* lunar phases.

According to the data in the literature NO donors do not have a significant effect on the behaviour of animals in the plus-maze test (Faria *et al.* 1997). However, NO precursor L-arginine reverses the anxiolytic effect of 7-NI in the plus-maze test (Yildiz *et al.* 2000).

NOS inhibitors also suppress isolation-induced ultrasounds (Campbell *et al.* 1999), induce an anxiolytic effect in other exploratory behavioural models — *e.g.* light-dark test, hole-board test (Calixto *et al.* 2001; Czech *et al.* 2003a) and have antidepressant-like effects in the forced swim test (Harkin *et al.* 1999).

In addition to their effects on behaviour NOS inhibitors have effect on hormone secretion (Budziszewska *et al.* 1999), metabolism (Uemura *et al.* 1997; Matsumoto *et al.* 1999), oxygen consumption (Gautier and Murariu 1999) thermoregulation (Carnio *et al.* 1999).

Since NO regulates numerous physiological processes, including neurotransmission, immune response, smooth muscle contractility and cell damage, NOS inhibitors have been proposed for the treatment of various diseases. Among possible indications for the use of NOS inhibitors, attenuation of opioid withdrawal (Vaupel *et al.* 1995), treatment of ethanol-induced excitotoxicity and ethanol dependence (Lancaster 1995) and treatment of chronic tension-type headache (Ashina *et al.* 1999) have been suggested.

1.3. NITRIC OXIDE AND ETHANOL EFFECTS

1.3.1. Discovery of interaction between ethanol and NO

By the end of the 1980s it was already well known that ethanol has profound effect on the glutamatergic system (for review see Allgaier 2002; Krystal *et al.* 2003). After the discovery of NO's participation in glutamatergic neurotransmission first studies concerning the interaction of L-arginine — NOS — NO pathways with ethanol were carried out. The effects of NO donors and NOS inhibitors on ethanol-induced gastric damage (MacNaughton *et al.* 1989), locomotor impairment (Khanna *et al.* 1993) and suppression of testosterone secretion (Adams *et al.* 1993) were demonstrated. After that a growing body of evidence has accumulated regarding the interaction of ethanol with NOergic pathways.

1.3.2. NO and acute ethanol administration

NOS inhibitors strengthen and NO donors attenuate the anaesthetic and toxic effects of ethanol after acute ethanol administration (Adams *et al.* 1994; Calapai *et al.* 1996).

In small doses (6 mg/kg) 7-NI increases the anxiolytic effect of acute ethanol administration (1 g/kg) (Ferreira *et al.* 1999).

NOS inhibitors 7-NI (Itzhak and Martin 2000) and L-NAME (Uzbay and Kayir 2003) block the effects of ethanol on locomotor activity.

NOS inhibitors also prevent the development of rapid tolerance to motor impairment caused by acute ethanol administration (Khanna *et al.* 1995).

1.3.3. NO and chronic ethanol administration

NOS inhibitors L-NOARG (Calapai *et al.* 1996), L-NAME (Rezvani *et al.* 1995) and 7-NI (Uzbay *et al.* 1998) reduce ethanol consumption in rats.

The nNOS is critically involved in neurobehavioral effects of alcohol. nNOS -/- mice showed an increased preference for ethanol (Spanagel *et al.* 2002).

Different authors have reported contradictory results concerning the effects of NOS inhibitors on ethanol withdrawal (for review see Uzbay and Oglesby 2001 and Table 1). Depending on the NOS inhibitor, its dose, animal species, strain, route of administration and evaluated signs, attenuation (Adams *et al.* 1995; Lallemand and De Witte 1997; Uzbay *et al.* 1997), worsening (Uzbay 2001) or no changes (Ikeda *et al.* 1999) in the severity of ethanol withdrawal signs have been described.

Table 1. Effects of NOS inhibitors on signs of ethanol withdrawal

Reference	Ethanol administration (species, route and duration)	NOS inhibitor (dose, route, regimen)	Effect
Adams *et al.* 1995	Rats, oral gavage for 3 days, final dose 9 g/kg/day	L-NAME 10–100 mg/kg, *i.p.*, acute	Attenuation of tremor, rigidity and hyperactivity, no effect on convulsions
Lallemand and De Witte 1997	Rats, inhalation for 30 days	L-NOARG 5 mg/kg, *i.p.*, chronic	Attenuation of hyperactivity
Uzbay *et al.* 1997	Rats, liquid diet for 16 days	L-NAME 30, 60 mg/kg and 7-NI 40, 80 mg/kg, *i.p.*, acute	Attenuation of audiogenic seizures and hyperactivity
Ikeda *et al.* 1999	Mice, liquid diet for 3,5 days	L-NOARG 5, 50 mg/kg, *s.c.*, repeated	No effect on hyperactivity, tremor or convulsions
Uzbay 2001	Rats, liquid diet for 26 days	L-NAME 50, 100, 200 mg/kg, *i.p.*, acute	Worsening of catatonia

1.3.4. Effect of ethanol on NO synthesis and NOS subtypes

Ethanol administration increases NO levels in plasma (Nanji *et al.* 2001; Baraona *et al.* 2002a). The effects of ethanol on NO production and NOS activity in organs depends on tissue, region, administration regimen and NOS subtype. Moreover, it has been demonstrated that ethanol-induced changes in NO production are gender-dependent (Spitzer and Spitzer 2000).

Acute ethanol administration decreases and chronic administration and withdrawal increases NO synthesis in neurons (Chandler *et al.* 1997; Czapski *et al.* 2002). Changes in NO synthesis differ significantly between separate brain regions (Naassila *et al.* 2003; Fitzgerald *et al.* 1995). For example, Fitzgerald *et al.* (1995) demonstrated that chronic ethanol administration decreased NOS activity in the cortex, hippocampus and striatum and increased it in *nucleus accumbens*.

Numerous studies have demonstrated that acute and chronic ethanol administration inhibit iNOS activity in glial cells (Wang *et al.* 1998), macrophages (Wakabayashi and Negoro 2002) and Kupffer cells (Kimura *et al.* 1996). Through its effect on iNOS ethanol can induce suppression of immune system (Wang *et al.* 1998) and liver injury (McKim *et al.* 2003).

NO mediates the complex effects of ethanol on endothelial function (for review see Puddey *et al.* 2001). Both activation (Venkov *et al.* 1999) and inhibition of eNOS (Oshita *et al.* 1994) have been reported. Acute ethanol administration also inhibits cGMP production in human platelets (Dong *et al.* 1995), possibly due to changes in NO synthesis (Bredt 2003). An increase in liver and coronary blood flow, caused by eNOS activation, has been considered beneficial (Baraona *et al.* 2002b) However, the increase in NO and reactive nitrogen intermediates has also been implicated in ethanol-induced organ damage, *e.g.* liver injury (Matsuda *et al.* 1999).

It must be noted that, like the effects of alcohols on receptors, their effects on NOS activity are dependent on chain length. Syapin *et al.* (1999) reported concentration-dependent iNOS inhibition from methanol to heptanol, and a significantly weaker effect with octanol and decanol.

1.3.5. NO and ethanol pharmacokinetics

Bulut *et al.* (1999) demonstrated that L-NAME inhibited the activity of alcohol dehydrogenase in gastric mucosa. However, no other studies had shown the effect of L-NAME on ethanol pharmacokinetics.

Gergel and Cederbaum (1996) demonstrated that NO inhibited alcohol dehydrogenase *in vitro*.

1.3.6. NO and ethanol-induced organ damage

It has also been demonstrated that NO-related agents have effect on ethanol-induced organ damage (for review see Lancaster 1992, 1995).

It must be stressed that the effects of NO-related agents on ethanol-induced organ damage are as contradictory as are data concerning the effects of NO-related drugs on ethanol withdrawal syndrome. Thus, protective effect against ethanol-induced damage to gastric mucosa has been reported both with NO (Konturek *et al.* 2003) and NOS inhibitors (Nahavandi *et al.* 2001). In the CNS the protective effect of NOS inhibitors against binge ethanol-induced brain damage has been reported (Zou *et al.* 1996).

1.3.7. NO and ethanol in humans

Data about the interaction of NO and ethanol in humans are scarce. Acute ethanol administration increases NO levels in blood (Matsuo *et al.* 2001). NO releasing substances in grape skins, *e.g.* polyphenols, have been proposed to be one of the reasons of "French paradox" — *i.e.* low mortality from coronary disease in comparison with that of other developed countries (Belleville 2002; Stanley and Mazier 1999). There are also data connecting fetal alcohol syndrome to changes in eNOS activity and generation of reactive oxygen species (Acevedo *et al.* 2001).

1.3.8. NO and other abused drugs

In addition to ethanol dependence nitric oxide pathways are involved in opioid dependence, psychostimulant dependence and nicotine dependence (for review see Uzbay and Oglesby 2001). Thus it has been demonstrated that NO mediates opioid withdrawal (Bhargava and Thorat 1996) and opioid tolerance (Dambisya and Lee 1996).

2. AIMS OF STUDY

The aim of our work was to further study the interaction of ethanol and L-arginine — NOS — NO pathways. For this purpose we observed the effects of NOS inhibitors 7-nitroindazole (7-NI), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NOARG) after acute and chronic ethanol administration and withdrawal.

The specific objectives were:

1. To study the effects of NOS inhibitors 7-NI, L-NAME and L-NOARG on the sedative and anesthetic effects of ethanol after acute ethanol administration.

3. To study the effects of NOS inhibitors after chronic ethanol administration and withdrawal.

2. To study the effects of NOS inhibitors on the pharmacokinetics of ethanol after acute and chronic ethanol administration.

4. To study the effects of NOS inhibitors on the toxicity of ethanol.

3. MATERIALS AND METHODS

3.1. ANIMALS

Naive male Wistar rats weighting 200–250 g and naive male balb/c mice weighing 30–35 g (Grindex Breeding Center, Riga, Latvia or Kuopio National Animal Centre, Kuopio, Finland) were used throughout the study. Rats and mice were maintained at constant conditions (temperature 20 ± 2°C; relative humidity 55 ± 5%) with water and standard laboratory food (commercial rat pellets Labfor R70, Lactamin, Stockholm, Sweden) available *ad libitum*. Lights were on from 7.00 a.m. to 7.00 p.m. For bedding aspen chips (chip size 4 × 4 × 1 mm, Tapvei, Kortteinen, Finland) were used.

3.2. DRUGS AND THEIR ADMINISTRATION

3.2.1. NOS inhibitors

7-NI, L-NAME L-NOARG were obtained from Sigma, St. Louis, MO, USA; diazepam was from La Roche, Basel, Switzerland. All drugs were suspended in saline with a few drops of Tween-80. Saline with a few drops of Tween-80 was used as a control vehicle. Drugs or vehicle were injected intraperitoneally (*i.p.*). Injection volume was 0.1 ml per 10 grams of body weight in mice and 0.1 ml per 100 grams of body weight in rats.

In experiments with acute ethanol administration drugs or vehicle were injected 30 min before ethanol.

In experiments with chronic ethanol administration drugs or vehicle were injected immediately or 6.5 hours after the removal of animals from the inhalation chamber.

3.2.2. Ethanol

In experiments with acute administration ethanol (96%) was diluted in saline and injected *i.p.* at a volume of 1 ml /100 g body weight.

For chronic ethanol administration we used method modified in our laboratory (Vassiljev *et al.* 1998) from the works of Sorg *et al.* (1996) and Ferko and Bobyock (1977). Mice or rats were placed into a plexiglas box, with standard laboratory food and water available *ad libitum*. Air was bubbled into a ethanol solution with air pump, and the vapour above the solution was passed through the chamber. To stabilise the vaporisation of ethanol the bottle with ethanol solution was placed into thermostat at a constant temperature. During the chronic administration the concentration of ethanol solution was gradually raised. Ethanol solution was changed twice a day (Fig. 1).

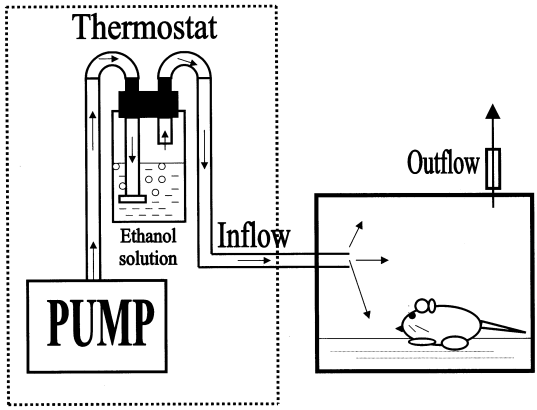

Figure 1. Schematic presentation of the inhalation chamber. Reprinted from Vassiljev *et al.* 1998.

This method produces high ethanol levels in blood and strong tolerance to and dependence on ethanol that manifests in the development of handling-induced convulsions after the end of ethanol administration.

3.3. BEHAVIOURAL METHODS

3.3.1. Measurement of sleeping time

Sleeping time was measured as the time elapsed between the loss and regaining of the righting reflex, the experimental criteria being that the animal had to regain its righting reflex 3 times within 1 min.

3.3.2. Open-field test

The open-field test was carried out according to the method modified from the works of Matto *et al.* (1997). The open-field of reduced size consisted of wood arena with dimensions 50 × 100 cm and 40 cm side walls. The surface of the floor of the arena was divided into eight squares of equal size. During 5 min the number of squares crossed and the number of rearings were recorded. On the basis of these data the total sum of exploratory events was calculated.

3.3.3. Ethanol withdrawal syndrome

The ethanol withdrawal syndrome was measured according to the method of Mead and Little (Mead and Little 1995). During the rating the mice were lifted up by the tail, turned first in one direction, then the other, then they were placed on cage top and observed.
Behavioural ratings:
1. Mild tremor on lifting and turning.
2. Continuous severe tremor on lifting and turning.
3. Clonic forelimb extensor spasm on lifting up.
4. Clonic forelimb extensor spasm on lifting, continued after placing mouse on cage top.

3.3.4. Plus-maze test

The plus-maze test was carried out according to Lister (Lister 1987). The plus-maze consisted of two open (8 × 17 cm) and two closed arms (8 × 17 × 30 cm), which were connected by a central platform (8 × 8 cm). Mice were placed on the central platform facing an open arm. During 5 minutes the number of entries made onto the open and into the closed arms and the time spent on the open arms were measured. On the basis of these data the percentage of entries made onto the open arms and the percentage of time spent on the open arms were calculated.

3.3.5. Staircase test

The staircase test was carried out according to the method modified from the works of previous authors (Simiand *et al*. 1984; Thiebot *et al*. 1973). The staircase was made of plastic and consisted of five identical steps 2.5 cm high, 10 cm wide and 7.5 cm deep. Staircase was surrounded by walls, the height of which was constant along the whole length of the staircase. Mouse was placed on the floor of the box with its back to the staircase. During a 3 min period the number of steps climbed and the number of rearings made were recorded.

3.4. MEASUREMENT OF WEIGHT CHANGES AND FOOD CONSUMPTION

For the measurement of weight changes rats were weighted twice a day for 14 days. Individual weight changes from the baseline (*i.e.* weight before the experiments) for each rat were calculated. Food consumption was evaluated based on the quantity of food left on cage tops after 24 hr.

3.5. MEASUREMENT OF ETHANOL CONCENTRATION IN BLOOD

Animals were killed by decapitation, trunk blood was collected and ethanol concentration in blood was measured by headspace gas chromatography with n-propanole as internal standard as reported by Goldbaum *et al.* (1966) or Solanky and Wylie (1993).

3.6. HISTOLOGICAL STUDIES

Rats were killed by decapitation and livers were collected for histological studies. The samples of tissue were fixed in 10% solution of neutral formalin and embedded in paraffin. After embedding in paraffin histological sections were made. Histological sections were stained with haematoxylin and eosin in routine use and with picro fuchsin and haematoxylin after van Gieson. To appreciate visually the preparations all fields of vision of the section were investigated by means of a microscope that was provided with an ocular network and a preparation shifter (obj. 40 X 0.65). Necrosis and connective tissue reaction were evaluated visually in 4 point scale where — stands for the absence of changes and +++ stands for prominent changes.

3.7. DATA ANALYSIS AND STATISTICS

All data were analysed by analysis of variance (ANOVA), using ethanol and drug treatment as factors. When appropriate the post-hoc statistical analysis was carried out. The minimum accepted level of statistical significance was at $P < 0.05$.

4. RESULTS

4.1. EFFECTS OF NOS INHIBITORS AFTER ACUTE ETHANOL ADMINISTRATION

4.1.1. Effects of NOS inhibitors on ethanol-induced sleep and ethanol ellimination in mice and rats

Ethanol administered at a dose of 3 g/kg (*i.p.*) induced sleep for 26.2 ± 11.1 min in vehicle-treated mice. 7-NI had significant effect on the duration of ethanol-induced sleep ($F_{4,27}$=3.95, $p<0.05$). Further analysis revealed that 7-NI administered *i.p.* at doses of 20–120 mg/kg 30 min before ethanol dose-dependently increased the duration of sleep, the effect being statistically significant at doses of 80 and 120 mg/kg (Fig. 2A).

5 min after administration, the ethanol concentration in the blood was 5.1 ± 0.1 mg/ml. In vehicle-treated mice blood ethanol levels decreased rapidly and 6 h later the blood ethanol level was zero. 7-NI dose-dependently inhibited ethanol clearance, the effect being statistically significant 6 h and 9 h after acute ethanol administration ($F_{4,10}$=166.4, $p<0.001$ and $F_{4,10}$=42.5, $p<0.001$, respectively) (Fig. 2B).

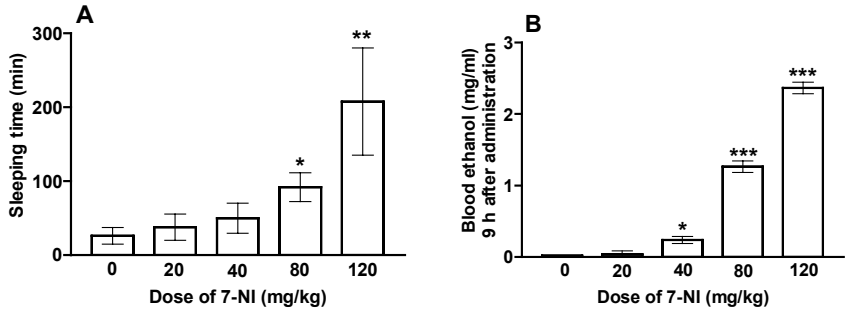

Figure 2. Effects of 7-NI on the duration of ethanol-induced sleep (A) and blood ethanol levels 9 hours (B) after acute ethanol administration. The data presented are means ± SEM from groups of 6–7 mice (ethanol-induced sleep) or 3 mice (blood ethanol levels).
* — $p<0.05$; ** — $p<0.01$; *** — $p<0.001$ vs. vehicle-treated mice (contrast analysis).

Ethanol at a dose of 2 g/kg (*i.p.*) did not induce sleep in vehicle-treated rats. However, the combined administration of ethanol (2 g/kg) and 7-NI at doses of 40, 80 and 120 mg/kg induced sleep for 49.4 ± 3.7 (n = 8), 204.0 ± 13.3 (n = 5) and 447.5 ± 62.8 minutes (n = 5), respectively.

L-NOARG at doses of 20 and 40 mg/kg significantly ($F_{2,14}$=12.47, $p<0.001$) increased the duration of sleep induced by the dose of ethanol 3 g/kg (*i.p.*), the effect being statistically significant at a dose of 40 mg/kg (Fig. 3).

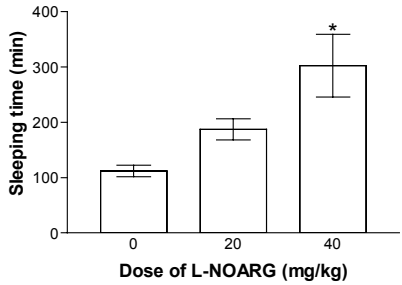

Figure 3. Effect of L-NOARG on the duration of ethanol-induced sleep in rats. The data presented are means ± SEM from groups of 6 rats.
* — $p<0.001$ vs. vehicle-treated rats (contrast analysis).

7-NI ($F_{1,14}=9.11$, $p<0.01$) and L-NOARG ($F_{1,14}=8.06$, $p<0.05$) at a dose of 20 mg/kg also significantly increased the duration of sleep caused by a higher dose (4 g/kg, *i.p.*) of ethanol (Fig. 4).

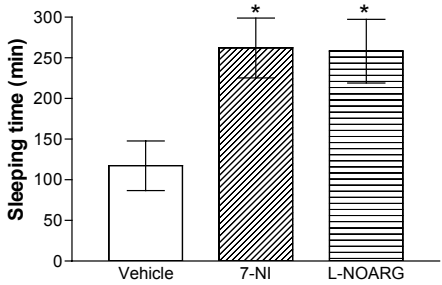

Figure 4. Effects of 7- -NI and L-NOARG on the duration of ethanol-induced sleep in rats. The data presented are means ± SEM from groups of 8 rats.
* — $p<0.05$ vs. vehicle-treated rats (Bonferroni test).

The combined administration of ethanol (4 g/kg) with L-NOARG (20 mg/kg) caused significant ($p<0.05$, Fischer's exact probability test) lethality during the first 3 days after the experiment. Whereas four of seven rats died in the L-NOARG-treated group, no deaths occured in vehicle- or 7-NI-treated rats.

After *i.p.* administration of ethanol at doses 2, 3 and 4 g/kg (Fig. 5 and Fig. 6) blood ethanol concentrations decreased rapidly. By 9 h after its acute administration, the ethanol concentration was nearly zero in vehicle-treated rats. L-NOARG at doses of 20 and 40 mg/kg had no effect on ethanol pharmacokinetics (Fig. 5).

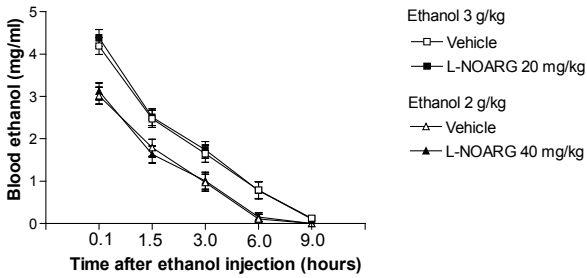

Figure 5. Blood-ethanol levels in vehicle- and L-NOARG-treated rats after acute ethanol administration. Data presented are means ± SEM from groups of 3 rats.

7-NI at doses 20 and 40 mg/kg had no effect on the pharmacokinetics of ethanol after acute administration (Fig. 6A). However, higher doses of 7-NI (80 and 120 mg/kg) significantly decreased ethanol clearance (Fig. 6B).

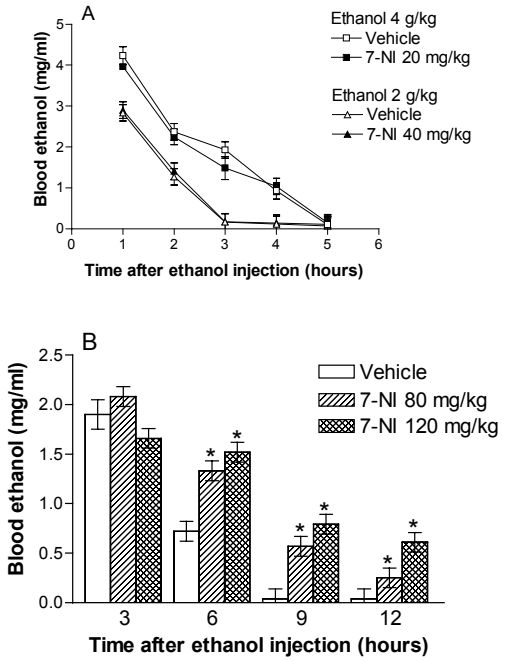

Figure 6. Blood ethanol levels in vehicle- and 7-NI-treated rats after acute ethanol administration.
(A) 7-NI was administered at doses of 20 and 40 mg/kg 30 min before ethanol (2 and 4 g/kg, *i.p.*).
(B) 7-NI was administered at doses of 80 and 120 mg/kg 30 min before ethanol (2 g/kg, *i.p.*)
Data presented are means ± SEM from groups of 3 rats.
* — $p<0.05$ vs. vehicle-treated rats (Bonferroni test).

4.1.2. Effects of ethanol and NOS inhibitors in open-field test in rats

Interaction of L-NOARG and ethanol in open-field test

Kruskal-Wallis one-way ANOVA showed significant effect of group on the number of squares crossed ($H=15.08$, $df=5$, $p<0.05$), on the number of rearings ($H=17.92$, $df=5$, $p<0.005$), and on the total number of exploratory events ($H=15.81$, $df=5$, $p<0.01$). Further analysis revealed that ethanol at a dose of 2 g/kg had no significant effect in the open-field of reduced size although it showed a trend towards decreasing exploratory activity (Table 2). L-NOARG dose-dependently decreased exploratory activity, the effect being significant with a dose of 40 mg/kg. However, the administration of L-NOARG (both at doses 20 and 40 mg/kg) 30 min before ethanol produced a profound decrease of exploratory activity as evidenced by a decrease in the number of squares crossed, the number of rearings and the total number of exploratory events (Table 2). It should be noted that the number of rearings was zero in rats treated with L-NOARG and ethanol.

Table 2. Effects of ethanol (2 g/kg), L-NOARG (20 and 40 mg/kg) and combined administration of ethanol and L-NOARG on the behaviour of rats in the open-field of reduced size

Group	n	Squares	Rearings	Total number
vehicle + vehicle	9	40.4 ± 3.1	24.0 ± 2.4	64.4 ± 5.3
vehicle + ethanol (2 g/kg)	9	28.0 ± 6.6	16.5 ± 5.2	44.5 ± 11.1
L-NOARG (20 mg/kg) + vehicle	4	27.0 ± 7.2	19.7 ± 6.4	46.7 ± 13.4
L-NOARG (40 mg/kg) + vehicle	4	19.2 ± 2.6*	4.7 ± 1.6*	24.0 ± 4.2*
L-NOARG (20 mg/kg) + ethanol (2 g/kg)	4	12.0 ± 2.0*	0.0 ± 0.0*	12.0 ± 2.0*
L-NOARG (40 mg/kg) + ethanol (2 g/kg)	4	8.0 ± 2.7*	0.0 ± 0.0*	8.0 ± 2.7*

Vehicle or L-NOARG were injected *i.p.* 30 min before vehicle or ethanol (2 g/kg, *i.p.*).
n — number of animals
* — $p<0.001$ vs. vehicle + vehicle group (Kolmogorov-Smirnov test)

Interaction of L-NAME and ethanol in open-field test

Kruskal-Wallis one-way analysis of variance showed significant effect of group on the number of squares crossed [H=15.0, df=5, p<0.01], on the number of rearings [H=35.3, df=5, p<0.01] and on the total number of exploratory events [H=18.0, df=5, p<0.01]. Further analysis revealed that ethanol at a dose of 2 g/kg decreased exploratory activity of rats in the open-field test as evidenced by decreased number of rearings and decreased number of exploratory effects (Table 3). L-NAME at a dose of 20 mg/kg had no effect and at a dose of 40 mg/kg decreased exploratory activity. The administration of L-NAME at a dose of 20 mg/kg 30 min before ethanol showed a trend towards decreasing exploratory activity as evidenced by a decrease in the number of squares crossed, the number of rearings and the total number of exploratory events. However, this tendency did not reach statistical significance as compared with rats who were treated only with ethanol. Surprisingly, the trend was not present with a dose of 40 mg/kg (Table 3).

Table 3. Effects of ethanol (2 g/kg), L-NAME (20 and 40 mg/kg) and combined administration of ethanol and L-NAME on the behaviour of rats in the open-field of reduced size

Group	n	Squares	Rearings	Total number
vehicle + vehicle	13	28.4 ± 4.2	15.9 ± 2.6	44.3 ± 6.6
vehicle + ethanol (2 g/kg)	13	18.2 ± 3.8	0.2 ± 0.1**	18.5 ± 3. 9*
L-NAME (20 mg/kg) + vehicle	6	33. 7 ± 3.8	13.8 ± 3.1	47.5 ± 6.8
L-NAME (40 mg/kg) + vehicle	7	9.3 ± 4.8*	5.0 ± 3.1*	14.3 ± 7.9*
L-NAME (20 mg/kg) + ethanol (2 g/kg)	6	10.3 ± 1.7**	0.0 ± 0.0**	10.3 ± 1.7**
L-NAME (40 mg/kg) + ethanol (2 g/kg)	7	32.0 ± 9.5	0.1 ± 0.1**	32.1 ± 9.6

Vehicle or L-NAME were injected *i.p.* 30 min before vehicle or ethanol (2 g/kg, i.p).
n — number of animals
* — p<0.05, ** — p<0.001 as compared with vehicle + vehicle group (Kolmogorov-Smirnov test).

4.2. EFFECTS OF NOS INHIBITORS AFTER CHRONIC ETHANOL ADMINISTRATION

4.2.1. Effects of 7-NI, L-NAME and L-NOARG on physical signs of ethanol withdrawal in mice

After the removal of mice from the inhalation chamber, parallel to a rapid fall in blood ethanol levels, the behavioural signs of ethanol withdrawal — severe tremor and convulsions — developed in mice. The expression of these signs was most pronounced 6–9 h after the end of ethanol administration (Fig. 7).

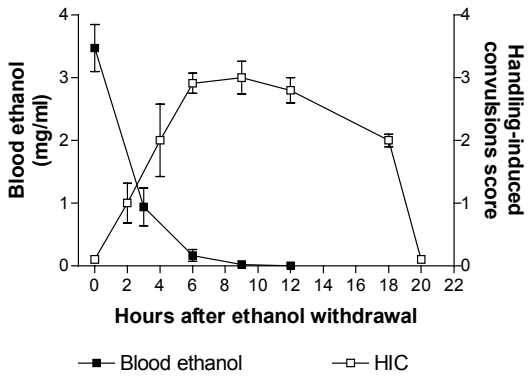

Figure 7. Disappearance of ethanol from blood of mice (y_1) and the development of signs of withdrawal (y_2) over a 20 h period. Data presented are means ± SEM from groups of 4 (blood levels) or 14 mice (handling-induced convulsions). Reprinted from Vassiljev *et al.* 1998.

Group had significant effect on handling-induced convulsions 6.5 h ($F_{9,50}$= 20.79, $p<0.001$) and 7.5 h ($F_{9,47}$=63.97, $p<0.001$) after the removal of mice from the inhalation chamber.

In accordance with previous data in the literature diazepam, used as a positive control drug in our experiments, at a dose of 5 mg/kg blocked the development of behavioural signs of ethanol withdrawal when administered before and eliminated them when administered during ethanol withdrawal (Table 4).

7-NI at a dose of 20 mg/kg blocked the development of the behavioural signs of ethanol withdrawal when administered immediately after the end of ethanol exposure, but had no effect when administered 6.5 hours later (Table 4).

L-NAME and L-NOARG administered at a dose of 20 mg/kg had no effect on ethanol withdrawal syndrome, irrespective of time of administration (Table 4).

In mice treated with 7-NI immediately after the end of ethanol exposure the fall of blood ethanol levels was slower, significant concentrations were measured in blood 7.5 hours after the end of ethanol exposure — 0.47 ± 0.03 mg/ml versus 0.09 ± 0.03 mg/ml in vehicle-treated mice. Two-way ANOVA showed significant effect of 7-NI ($F_{1,16}$=15.26, $p<0.01$) and injection time ($F_{1,16}$=22.95, $p<0.01$) on blood ethanol levels. Diazepam, L-NAME and L-NOARG had no effect on blood ethanol levels.

Table 4. Effects of diazepam, 7-NI, L-NAME and L-NOARG on the physical signs of ethanol withdrawal

Drug	Administration time	n	Handling-induced convulsions	
			6.5 h after withdrawal	7.5 h after withdrawal
Vehicle	0	10	2.90 ± 0.10	2.78 ± 0.15
	6.5	10	2.70 ± 0.21	2.90 ± 0.10
Diazepam	0	5	0.00 ± 0.00*	0.00 ± 0.00*
	6.5	5	2.60 ± 0.24	0.00 ± 0.00*
7-NI	0	5	0.80 ± 0.20*	0.80 ± 0.20*
	6.5	5	2.60 ± 0.24	2.80 ± 0.20
L-NAME	0	5	2.80 ± 0.20	3.00 ± 0.00
	6.5	5	3.00 ± 0.00	2.80 ± 0.20
L-NOARG	0	5	2.40 ± 0.40	3.25 ± 0.25
	6.5	5	2.80 ± 0.20	2.80 ± 0.20

Administration time: 0 — immediately after the end of ethanol administration; 6.5 — 6.5 h after the end of ethanol administration
Data presented are means ± SEM
n — number of animals
* — $p<0.01$ vs. corresponding vehicle-treated group (contrast analysis)

4.2.2. Effect of 7-NI on anxiogenic effect of ethanol withdrawal in mice in plus-maze and staircase tests

In control mice 7-NI at a dose of 20 mg/kg, administered *i.p.* 60 min or 7.5 h before the plus-maze test, induced an anxiolytic effect as evidenced by an increase in the number of entries made onto the open arms ($F_{2,13}$=4.24, $p<0.05$), in the percentage of entries made onto the open arms ($F_{2,13}$=7.84, $p<0.01$) and in the percentage of time spent on the open arms ($F_{2,13}$= 4.85, $p<0.05$). 7-NI had no effect on the total number of entries made (Table 5).

Chronic ethanol administration caused an anxiolytic effect as evidenced by an increase in the number of entries made onto the open arms, in the percentage

of entries made onto the open arms and in the percentage of time spent on the open arms. Chronic ethanol administration also increased the total number of entries made.

The administration of 7-NI during ethanol administration at a dose of 20 mg/kg caused a decrease in the number of entries made onto the open arms ($F_{1,8}$=5.57, $p<0.05$), in the total number of entries made in the plus-maze ($F_{1,8}$=5.83, $p<0.05$), in the percentage of entries made onto the open arms ($F_{1,8}$=6.59, $p<0.05$) and in the percentage of time spent on the open arms ($F_{1,8}$=5.79, $p<0.05$) (Table 5).

Ethanol withdrawal caused an anxiogenic effect as evidenced by a decrease in the number of entries made onto the open arms, in the percentage of entries made onto the open arms and in the percentage of time spent on the open arms. The total number of entries was also decreased in ethanol-withdrawn mice. The administration of 7-NI immediately after the end of ethanol exposure or 6.5 h later had no effect on the behaviour of ethanol-withdrawn mice (Table 5).

Table 5. Effects of 7-NI on the behaviour of control, ethanol-intoxicated (ethanol) and ethanol-withdrawn (withdrawn) mice in the plus-maze test

Group	n	Entries made onto open arms	Total number of Entries made	% Entries made onto open arms	% Time spent on open arms
Control/vehicle	6	3.8 ± 0.5	17.2 ± 1.2	23.1 ± 3.6	4.4 ± 0.8
Control/7-NI 60 min before test	5	9.6 ± 2.3*	20.2 ± 3.6	45.8 ± 5.7**	17.2 ± 4.7**
Control/7-NI 7.5 h before test	5	6.6 ± 1.1	15.2 ± 1.8	42.6 ± 4.2**	12.7 ± 2.8*
Ethanol/vehicle	6	16.0 ± 2.4**	27.2 ± 2.9**	57.5 ± 3.2**	27.1 ± 3.7**
Ethanol/7-NI 60 min before test	5	6.8 ± 3.1++	16.8 ± 2.9++	35.4 ± 9.6++	12.2 ± 5.3++
Withdrawn/vehicle	6	6.2 ± 1.3++	15.8 ± 1.8++	39.4 ± 6.1+	10.8 ± 2.8++
Withdrawn/7-NI 60 min before test	5	8.0 ± 1.8	18.8 ± 2.5	41.7 ± 5.3	11.5 ± 2.3
Withdrawn/7-NI 7.5 h before test	5	3.6 ± 0.7	11.8 ± 2.4	32.0 ± 3.5	6.5 ± 1.0

The data presented are means ± SEM.
n — number of animals.
* — $p<0.05$, ** — $p<0.01$ vs. control/vehicle;
+ — $p<0.05$, ++ — $p<0.01$ vs. ethanol-intoxicated/vehicle (contrast analysis).

7-NI administered at a dose of 20 mg/kg 60 min or 7.5 h before the staircase test had no effect on the number of steps or rearings made by control mice (Table 6). However, when administered 7.5 h before the staircase test 7-NI induced a tendency towards decreasing the number of rearings ($p=0.051$).

Chronic ethanol administration significantly increased the exploratory activity of mice in the staircase as evidenced by an increased number of steps. The administration of 7-NI during ethanol administration at a dose of 20 mg/kg caused a decrease in the number of steps ($F_{1,8}$=11.35, $p<0.05$) and rearings ($F_{1,8}$=17.73, $p<0.005$] (Table 6).

Ethanol withdrawal significantly decreased the number of steps and had no effect on the number of rearings. 7-NI administered at a dose of 20 mg/kg 60 min or 7.5 h before the staircase test had no effect on the behaviour of ethanol-withdrawn mice (Table 6).

Immediately after the end of behavioural experiments (*i.e.* 10 min after the removal of mice from the inhalation box) mean blood ethanol concentration was 1.66 ± 0.20 mg/ml (n = 6) in ethanol-intoxicated group. After the end of ethanol administration blood ethanol levels decreased rapidly and 7.5 h later it was practically zero in all groups withdrawn from ethanol. Contrary to our previous experiments, the administration of 7-NI at a dose of 20 mg/kg immediately after the end of ethanol exposure had no effect on blood ethanol levels 7.5 h later as compared with vehicle-treated mice.

Table 6. Effects of 7-NI on the behaviour of control, ethanol-intoxicated (ethanol) and ethanol-withdrawn (withdrawn) mice in the staircase test

Group	n	Steps	Rearings
Control/vehicle	6	63.2 ± 6.1	19.2 ± 1.5
Control/7-NI 60 min before test	5	52.2 ± 3.5	19.6 ± 3.0
Control/7-NI 7.5 h before test	5	48.6 ± 8.6	13.8 ± 1.8
Ethanol/vehicle	6	85.2 ± 9.6*	20.3 ± 1.5
Ethanol/7-NI 60 min before test	5	43.5 ± 3.6+	8.3 ± 2. 8+
Withdrawn/vehicle	6	51.5 ± 8.5+	19.7 ± 4.0
Withdrawn/7-NI 60 min before test	5	51.4 ± 4.1	20.8 ± 1.4
Withdrawn/7-NI 7.5 h before test	5	37.6 ± 5.9	17.2 ± 3.6

Data presented are means ± SEM.
n — number of animals
* — $p < 0.05$ vs. control/vehicle;
+ — $p < 0.01$ vs. ethanol-intoxicated/vehicle (contrast analysis).

4.2.3. Effect of 7-NI on ethanol pharmacokinetics after chronic administration to rats

Immediately after the removal of rats from the inhalation chamber ethanol concentration in blood was 2.39 ± 0.10 mg/ml, in vehicle-treated rats blood ethanol levels decreased rapidly and already 6 h after withdrawal ethanol levels were negligible (Fig 8.). In 7-NI-treated rats the fall in ethanol concentrations

was significantly slower ($F_{1,22}$=11.12, p<0.01), ethanol levels were measured even 12 hours after the end of ethanol exposure (Fig. 8).

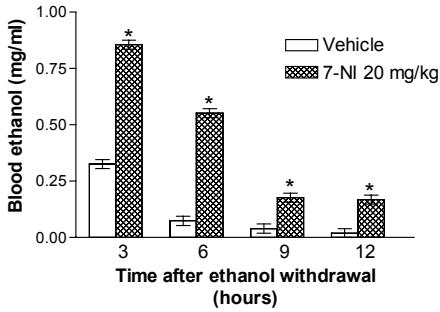

Figure 8. Blood ethanol levels in vehicle- and 7-NI-treated rats after chronic ethanol administration. 7-NI was administered immediately after the end of ethanol exposure (18 days by inhalation). Data presented are means ± SEM from groups of 3 rats.
* — $p < 0.05$ vs. vehicle-treated rats (Bonferroni test).

4.3. EFFECTS OF NOS INHIBITORS ON LONG-TERM TOXICITY AND HISTOLOGICAL CHANGES

4.3.1. Changes in body weight and food consumption

After the administration of ethanol (2 g/kg) and L-NOARG (20 and 40 mg/kg) and the open-field test the weight of animals was recorded for two weeks. The weight of vehicle-treated rats increased gradually. Ethanol and L-NOARG did not induce significant changes in body weight as compared with vehicle-treated rats (Fig. 9A). However, the combined administration of L-NOARG and ethanol caused a significant decrease in body weight that lasted for 14 days. At the dose of 40 mg/kg this effect was more pronounced (Fig. 9A).
Food consumption did not differ among groups (Fig. 9B).

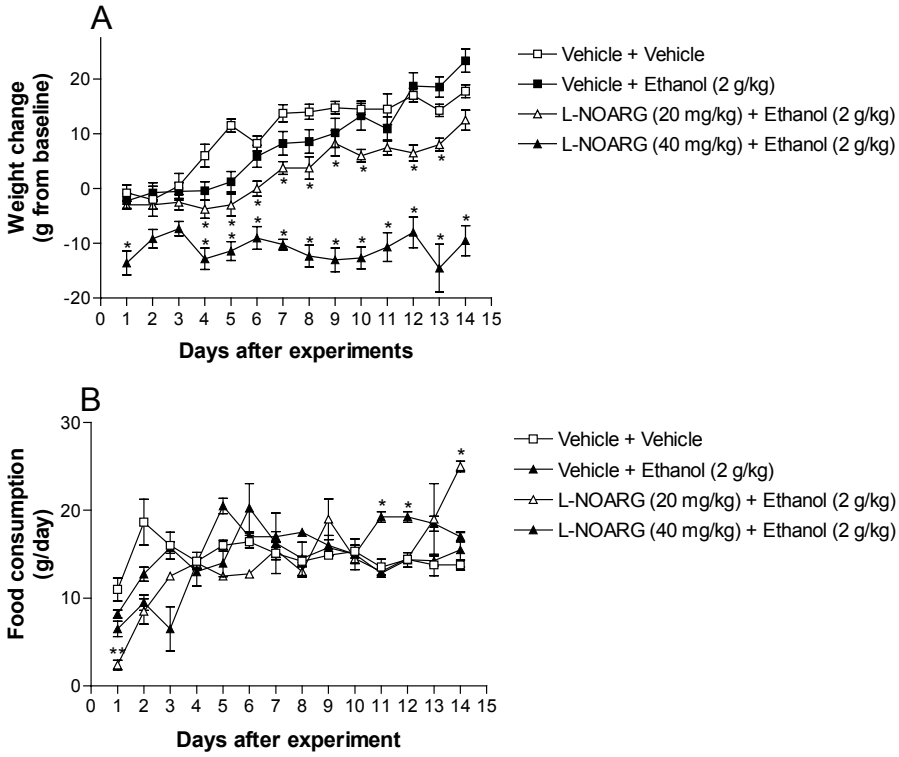

Fig. 9. Changes in the weight of rats (A) and food consumption (B) during 14 days after the administration of vehicle, ethanol or the combination of L-NOARG and ethanol. Data presented are means ± SEM from groups of 4-9 rats.
* — $p < 0.01$ vs. vehicle + vehicle group (contrast analysis)

4.3.2. Results of histological studies

During the observation of rats for weight changes some of the rats were sacrificed for histological studies. No pathological changes were observed in the livers of rats treated only with vehicle or L-NOARG (20 and 40 mg/kg). In rats treated with ethanol (2 g/kg) already on the first day after experiment liver cell injury could be seen. The cells were swelled and loss of the cellular outline and nuclear staining took place. The cells appeared to undergo fragmentation and condensation. Necrosis of clusters of liver cells was seen. Sinusoids were wide due to hyperemia. On the seventh day necrotic areas were very small. Cellular swelling with cellular disarray took place. In the portal tract and around the blood vessels an inflammatory infiltrate could be seen. On the fourteenth day there were only some necrotic cells but foci of infiltrate with macrophages and

fibroblasts could be found. On the fourteenth day lipidic degeneration also took place in hepatocytes in small amount (Table 7, Fig. 10).

In rats treated with L-NOARG (40 mg/kg) before the administration of ethanol (2 g/kg) there were no prominent differences as compared with rats treated with vehicle before the administration of ethanol. Only the reaction of connective tissue, as evidenced by fascicles and nidi of fibroblasts, was more pronounced on the seventh day (Table 7, Fig. 10)

Table 7. Effects of L-NOARG (40 mg/kg) on hepatic necrosis and connective tissue reaction (CTR) caused by the acute administration of ethanol (2 g/kg, *i.p.*)

	Vehicle + ethanol	L-NOARG + ethanol
Day 1	necrosis +++ CTR —	necrosis +++ CTR —
Day 7	necrosis ++ CTR —	necrosis ++ CTR ++
Day 14	necrosis + CTR ++	necrosis + CTR ++

Necrosis and connective tissue reaction were evaluated visually in 4 point scale (– - absence of changes, +++ - prominent changes).

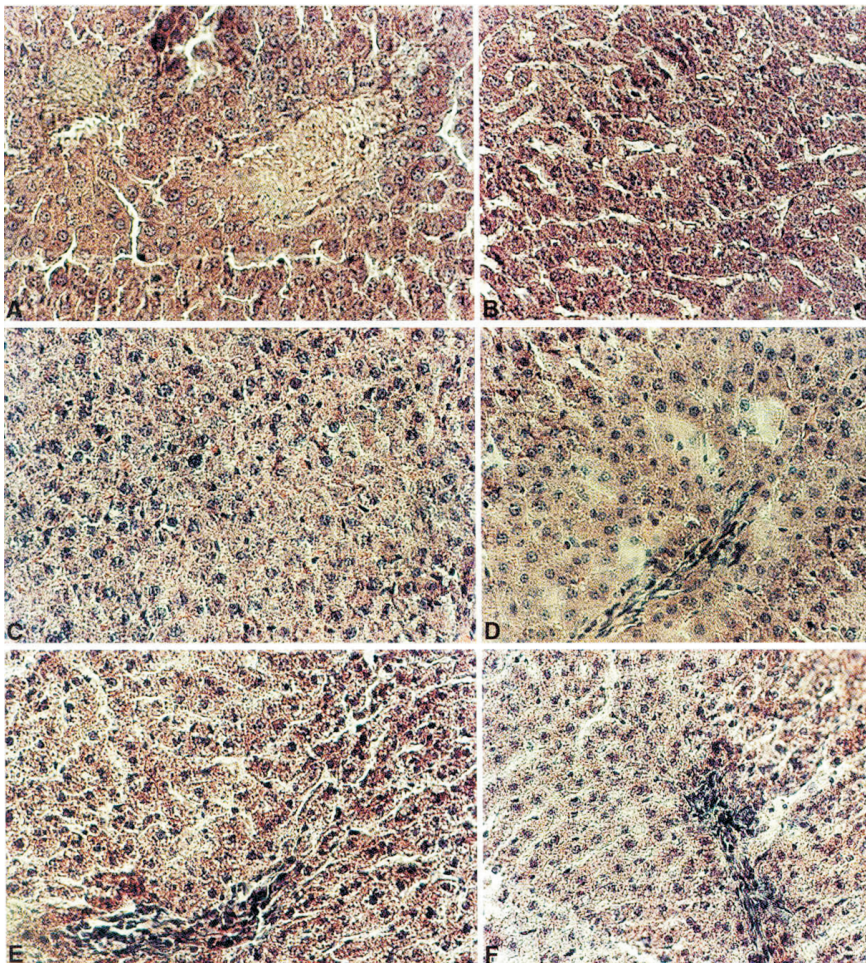

Figure 10. Effects of L-NOARG (40 mg/kg, *i.p.*) on hepatic necrosis and co
tissue reaction caused by the acute administration of ethanol (2 g/kg, *i.p.*). L-N
was injected *i.p.* 30 min before the acute administration of ethanol. 40
magnification, haematoxylin and eosin staining.
A — The first day after vehicle + ethanol administration. Necrotic areas with th
cellular outline can be seen. Nuclei of the cells have disappeared — caryolysis h
place.
B — The first day after L-NOARG + ethanol administration. Necrotic hepatoc
be seen with caryolysis and cytolysis.
C — The seventh day after vehicle + ethanol administration. Disarray of the
cells has taken place.
D — The seventh day after L-NOARG + ethanol administration. Some necrotic
be seen. Reaction of connective tissue is observed as the result of organis
necrosis.
E — The fourteenth day after vehicle + ethanol administration. Focus of epitheli
and fibroblasts.
F — The fourteenth day after L-NOARG + ethanol administration. Connectiv
fascicle with fibroblasts and fibrocytes are seen.

5. DISCUSSION

5.1. EFFECTS OF NOS INHIBITORS AFTER ACUTE ETHANOL ADMINISTRATION

In our experiments NOS inhibitors 7-NI and L-NOARG significantly increased the duration of ethanol-induced sleep in mice and rats. These data are in accordance with previous data in the literature where NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) increased the duration of ethanol-induced sleep (Adams *et al.* 1994). Synergistic CNS depression was also observed in the open-field test, where the co-administration of ethanol with NOS inhibitors L-NAME and L-NOARG caused profound sedative effect.

However, the enhancement of the sedative effect of ethanol was not present with L-NAME's dose of 40 mg/kg. This was unexpected since L-NAME significantly enhanced ethanols anesthetic effect. One possible explanation of this phenomenon is that the coadministration of L-NAME at a dose of 40 mg/kg and ethanol at a dose of 2 g/kg causes strong stress. Depending on the character of stressor and the duration of stress, stress can increase the locomotor activity of rats (Hall *et al.* 1998; Stohr *et al.* 1999).

In accordance with previous data in the literature (Adams et *al.* 1994; Calapai *et al.* 1996; Khanna *et al.*, 1995) L-NOARG and L-NAME had no effect on ethanol pharmacokinetics after acute or chronic ethanol administration.

Our experiments also showed that lower doses of 7-NI (20 and 40 mg/kg) had no effect on ethanol pharmacokinetics after acute ethanol administration to rats.

Therefore, changes in the effects of ethanol, caused by lower doses of 7-NI and by L-NAME and L-NOARG are not due to pharmacokinetic changes.

7-NI had pronounced effect on ethanol pharmacokinetics after chronic ethanol administration. One possible explanation is that this effect is caused by the interaction of ethanol and NO in the liver. This hypothesis is supported by data in the literature showing significant interaction between the effects of ethanol, NO and NOS inhibitors on hepatic microcirculation and blood flow (Oshita *et al.* 1993). However, the hypothesis that changes in ethanol pharmacokinetics caused by 7-NI are due to its effect on NOS is contradicted by several facts. First, a dose of 7-NI 20 mg/kg that causes inhibition of NOS activity by 90% (Connop *et al.* 1994) had no effect on ethanol pharmacokinetics after acute administration. Secondly, it has been shown that NO inhibits the catalytic activity of alcohol dehydrogenase in rat hepatocytes (Gergel and Cederbaum, 1996). Therefore an inhibition of NOS could hardly inhibit alcohol metabolism. Third, other NOS inhibitors do not influence ethanol pharmacokinetics. Therefore it could be presumed that this effect is not caused by 7-NI's effect on NOS, but by some other mechanisms. Most likely this effect is caused by 1,2-diazole ring in 7-NI's chemical structure. Pyrazole (1,2-diazole), which resembles 7-NI

(Fig. 11) has been shown to inhibit alcohol dehydrogenase (Ferko and Bobyock 1977).

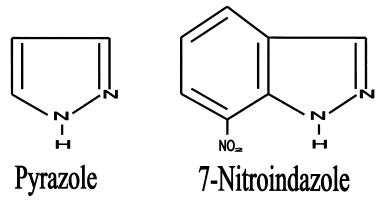

Figure 11. Structural formulae of pyrazole and 7-nitroindazole (7-NI).

It is an complicated question why 7-NI had more pronounced effect on ethanol pharmacokinetics after chronic ethanol administration both in mice and rats. One possible explanation is that prolonged exposure to high blood ethanol concentrations causes hepatic damage and changes in the metabolism of ethanol or/and 7-NI in the liver, thereby causing increased sensitivity of animals to combined administration of these drugs. In mice, continuously exposed to ethanol vapour, fatty change in the liver and lesions resembling those of alcoholic hepatitis in man are observed already on 2–5 day of exposure (Goldin and Wickramasinghe 1987).

NOS inhibitors, especially 7-NI, have been proposed in the literature as candidates for use as attenuators of opioid withdrawal (Vaupel *et al.* 1995) and also as possible treatments for ethanol-induced excitotoxicity and ethanol dependence (Lancaster 1995). However, on the basis of our data serious pharmacodynamic and/or pharmacokinetic interactions between NOS inhibitors and ethanol are possible.

5.2. EFFECTS OF NOS INHIBITORS AFTER CHRONIC ETHANOL ADMINISTRATION

7-NI significantly attenuated the behavioural signs of ethanol withdrawal and slowed ethanol clearance after chronic administration. It can be proposed that the effect of 7-NI on ethanol withdrawal syndrome is caused by changes in ethanol pharmacokinetics. This hypothesis is supported by the fact that 7-NI blocked the development of the behavioural signs of withdrawal when administered before, but not during withdrawal. At the same time diazepam, which alleviates ethanol withdrawal symptoms by facilitating GABAergic transmission (Ticku *et al.* 1983), had effect also after the development of withdrawal syndrome.

This hypothesis is also supported by the fact that other NOS inhibitors L-NAME and L-NOARG at doses that inhibit NOS activity in the brain by at least

80% (Salter *et al.* 1995) had no effect on the behavioural signs of ethanol withdrawal when given immediately after the end of ethanol exposure or during withdrawal.

These data contradict previously reported results of Uzbay *et al.* (Uzbay *et al.* 1997) showing that L-NAME administered at doses of 30 and 60 mg/kg immediately and 6 h after the end of ethanol exposure alleviated the signs of ethanol withdrawal in rats. This discrepancy might be explained by differences in used animal species (rats *vs.* mice) and methods for assessment of ethanol withdrawal (audiogenic convulsions vs handling-induced convulsions). Also, the doses used by Uzbay *et al.* (1997) (30 mg/kg twice during withdrawal) were significantly higher than those in our study.

In control mice 7-NI induced an anxiolytic effect as evidenced by an increase in the percentage of entries made onto the open arms and the percentage of time spent on the open arms of the plus-maze (Pellow *et al*. 1985; Lister 1987). These results also agree with previous studies demonstrating the anxiolytic effect of 7-NI in the plus-maze test (Volke *et al*. 1997; Yildiz *et al*. 2000). However, a novel finding of our studies is that the anxiolytic effect of 7-NI is long-lasting and is observed even 7.5 h after its acute administration. This finding is interesting considering the time course of NOS inhibition by 7-NI (MacKenzie *et al*. 1994) with maximal inhibition of NOS activity in striatum, cerebellum, hippocampus, cerebral cortex, and olfactory bulb 0.5 h after *i.p.* administration with consequent fast recovery of NOS activity and absence of any changes 4 h later. The possible explanation is that the inhibition of NOS in the brain triggers a chain of neurochemical reactions causing long-lasting behavioural effects.

In accordance with numerous data in the literature chronic ethanol administration induced an anxiolytic and ethanol withdrawal — an anxiogenic effect in the plus-maze test (Onaivi *et al*. 1989; Cole *et al*. 2000; File *et al*. 1993). In ethanol-intoxicated mice the administration of 7-NI caused a strong sedative effect that was evidenced by a decrease in the number of entries made onto the open arms and in the total number of entries. As a consequence the percentage of entries made onto the open arms and the percentage of time spent on the open arms were also decreased. These results contradict those of Ferreira *et al*. (1999) who reported that 7-NI increased the percentage of open arm entries and time spent on open arms in rats injected with ethanol. This discrepancy can be explained with different routes and regimens of ethanol administration used — Ferreira *et al*. (1994) used acute ethanol administration by *i.p.* injection while we used chronic ethanol administration by inhalation. Therefore it could be assumed that the administration of 7-NI could cause strong synergistic CNS depression with ethanol observed also in experiments concerning the duration of ethanol-induced sleep. The administration of 7-NI had no effect on the behaviour of ethanol-withdrawn mice in the plus-maze test.

7-NI did not significantly affect the number of steps or rearings made by control mice in the staircase test.

Chronic ethanol administration increased the number of steps made by mice in the staircase test. In the earlier works, regarding the staircase test, rearing was considered an index of the anxiety or emotionality and climbing (the number of steps) — an index of exploratory or locomotor activity (Simiand *et al*. 1973; Thiebot *et al*. 1984). However, it has also been proposed that changes observed in the number of rearings rather reflect changes in the level of locomotor activity (Lister 1990). It is probable that both of them are indices of exploratory behaviour and both of them depend on the level of anxiety and the level of locomotor activity. Therefore it could be concluded that chronic ethanol administration increases the locomotor activity of mice in the staircase test. However, the effects of chronic ethanol administration in the staircase test differ from the effects of acute ethanol administration. It had been reported in the literature that acute ethanol administration reduces the number of rearing at doses that does not influence the number of steps climbed (Pollard and Howard 1986; Belzung *et al*. 1988). Likewise the plus-maze test, the administration of 7-NI to ethanol-intoxicated mice caused prominent sedative effect that was evidenced by a decrease in the number of steps and rearings made in the staircase test.

Ethanol withdrawal decreased the number of steps made in the staircase test. These results agree with those of Moy *et al.* (1997) who reported a decrease of locomotor activity in the plus-maze test after withdrawal chronic ethanol administration. 7-NI had no effect on the behaviour of ethanol-withdrawn mice in the staircase test.

It must be noted that 7-NI did not have effect on ethanol pharmacokinetics in experiments concerning the anxiogenic effect of ethanol withdrawal in the plus-maze and staircase tests. The most probable cause of this discrepancy lies in different strains of mice used. In experiments concerning physical dependence we used balb/c mice and in experiments concerning anxiogenic effect of ethanol withdrawal we used NIH/S mice. It is possible that these strains differ in their sensitivity to the effects of 7-NI.

In conclusion, 7-NI had no effect on the behavioural changes caused by ethanol withdrawal in the plus-maze and staircase test. Therefore it can be proposed that NOergic pathways do not have a major role in the behavioural changes caused by ethanol withdrawal. At the same time NOS inhibitors can cause synergistic CNS depression with ethanol.

5.3. EFFFECTS OF NOS INHIBITORS ON LONG-TERM TOXICITY AND HISTOLOGICAL CHANGES

L-NOARG also significantly increased the toxic effect of ethanol that was evidenced by significant increase in post-experimental lethality and a significant decrease of body weight. In accordance with previous data (Czech 1996) L-NOARG had no effect on food intake itself or in combination with ethanol. The

causes of this effect are unclear, however an attenuation of ethanol-induced organ degeneration (*e.g.* liver damage) could be assumed. This assumption is supported by data in the literature showing that NOS inhibitors L-NAME and 7-NI significantly increase ethanol-dependent neuronal degeneration (Zou *et al.* 1996).

However, there were no prominent changes in the degree of necrosis between rats treated with vehicle and rats treated with L-NOARG before the administration of ethanol, only connective tissue reaction was more pronounced on the seventh day after the experiment. Therefore it is hard to determine the importance of hepatic damage in weight loss caused by co-administration of L-NOARG and ethanol.

CONCLUSIONS

1. NOS inhibitors 7-NI, L-NAME and L-NOARG increased the sedative and anesthetic effects of ethanol as evidenced by an increase of the ethanol-induced sleep and sedative effect in the open-field test. These results suggest a role of L-arginine — NOS — NO pathways in the acute effects of ethanol.

2. 7-NI inhibited the elimination of ethanol. This effect is not probably due to 7-NIs effect on NO synthesis but by the presence of 1,2-diazole ring in 7-NIs chemical structure, causing inhibition of alcohol dehydrogenase.

3. L-NAME and L-NOARG did not have effect on the physical signs of ethanol withdrawal and 7-NI had attenuated them only due to pharmacokinetic interaction. However, 7-NI did not have a significant effect on the anxiogenic and locomotor depressant effects of ethanol in the plus-maze and staircase tests. On the basis of these data it could be proposed that L-arginine — NOS — NO pathways do not have a prominent role in ethanol withdrawal syndrome.

4. The coadministration of ethanol and L-NOARG induced a long-term toxicity as evidenced by increased post-experimental lethality and decrease in body weight observed during 14 days. The basis of this could be potentiation of ethanol-induced organ toxicity.

5. NOS inhibitors have been proposed in the literature as possible treatments for ethanol-induced excitotoxicity and ethanol dependence. However, on the basis of our data serious pharmacodynamic and/or pharmacokinetic interactions with ethanol are possible.

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SUMMARY IN ESTONIAN

Influence of nitric oxide syntase inhibitors on the effects of ethanol after acute and chronic ethanol administration and withdrawal

Kirjanduses on andmeid selle kohta, et etanooli toimed on osaliselt vahendatud L-arginiini — lämmastikoksiidi süntaasi (NOS) — NO ülekandeteede kaudu. Meie töö eesmärgiks oli etanooli ja NOergiliste ülejuhteteede interaktsiooni uurimine. Selleks jälgiti NOS inhibiitorite 7-nitroindasooli (7-NI), N^G-nitro-L-arginiini metüülestri (L-NAME) ja N^G-nitro-L-arginiini (L-NOARG) toimet pärast etanooli akuutset ning kroonilist manustamist ja võõrutust.

Katsetes, mis puudutasid etanooli akuutseid toimeid, manustati etanooli intraperitoneealselt (i.p.) 30 min. enne käitumuslikke teste (avarväli, pluss-puuri test) või vahetult enne etanoolist-põhjustatud une kestuse mõõtmist. Nendes katsetes manustati NOS inhibiitoreid i.p. 30 min. enne etanooli — s.t. 60 min. enne katseid.

Krooniliseks etanooli manustamiseks asetati hiired inhalatsiooni kambrisse, kus päev-päevalt suurendati etanooli kontsentratsiooni sissehingatavas õhus. Erinevates gruppides jälgiti etanooli võõrutuse füüsilisi nähte või anksiogeenset toimet pluss-puuris ja trepptestis 6,5 või 7,5 h pärast manustamise lõppu. NOS inhibiitoreid manustati i.p. vahetult pärast etanooli manustamise lõppu või 6,5 h hiljem.

NOS inhibiitorid 7-NI (20–120 mg/kg), L-NAME (20, 40 mg/kg) ja L-NOARG (20, 40 mg/kg) pikendasid oluliselt etanoolist (2, 3, 4 g/kg) põhjustatud une kestust hiirtel ja rottidel. L-NAME (20, 40 mg/kg) ja L-NOARG (20, 40 mg/kg) tugevdasid samuti oluliselt etanooli (2 g/kg) sedatiivset toimet rottidel avarvälja testis. L-NAME ja L-NOARG ei avaldanud toimet etanooli farmakokineetikale. 7-NI väikestes doosides (20, 40 mg/kg) ei avaldanud toimet ja suurtes doosides (80, 40 mg/kg) pärssis etanooli elliminatsiooni.

Pärast hiirte eemaldamist inhalatsiooni kambrist arenesid neil paralleelselt etanooli taseme kiire langusega võõrutusnähud — treemor ja krambid. 7-NI, manustatuna doosis 20 mg/kg vahetult pärast etanooli manustamise lõppu, blokeeris võõrutusnähtude arengu, kuid ei avaldanud toimet manustatuna pärast nähtude väljakujunemist. L-NAME ja L-NOARG doosis 20 mg/kg ei avaldanud toimet etanooli võõrutusnähtudele olenemata manustamise ajast. 7-NI manustamine vahetult pärast etanooli manustamise lõppu põhjustas etanooli elliminatsiooni olulise pärssimise, ka 7,5 h pärast manustamise lõppu esinesid veres kõrged etanooli kontsentratsioonid.

7-NI doosis 20 mg/kg avaldas pluss-puuri testis anksiolüütilist toimet. Krooniline etanooli manustamine avaldas pluss-puuri testis anksiolüütilist ja etanooli võõrutus — anksiogeenset toimet. 7-NI ei avaldanud toimet etanooli-joobes hiirte käitumisele. Samuti ei avaldanud 7-NI toimet etanooli võõrutuse

anksiogeensele ja lokomotoorset aktiivsust pärssivale toimele pluss-puuris ja trepp testis.

14 päeva jooksul peale avarvälja testi jälgiti rottide kehakaalu ja toidu tarbimist. Ilmnes, et etanooli (2 g/kg) ja L-NOARGi (20 ja 40 mg/kg) kombineeritud manustamine põhjustas rottide kehakaalu olulise languse. Histoloogilised uuringud ei näidanud etanoolist-põhjustatud maksakahjustuse olulist tugevnemist.

Järeldused

1. NOS inhibiitorid 7-NI, L-NAME ja L-NOARG tugevdasid etanooli sedatiivset ja anesteetilist toimet, mis väljendus etanoolist-põhjustatud une kestuse pikenemises ja sedatiivses toimes avarväljas. Need tulemused viitavad L-arginiini — NOS — NO ülekandeteede osalusele etanooli akuutsetes efektides.
2. 7-NI pidurdas etanooli elliminatsiooni. See toime ei ole tõenäoliselt seotud 7-NI toimega NO sünteesile vaid on põhjustatud 7-NI keemilises struktuuris sisalduvast 1,2-diasooltuumast.
3. L-NAME ja L-NOARG ei avaldanud toimet etanooli võõrutuse füüsilistele nähtudele ja 7-NI pärssis võõrutussündroomi füüsilisi nähte ainult tingituna farmakokineetilisest interaktsioonist ja etanooli elliminatsiooni aeglustumisest. 7-NI ei avaldanud toimet etanooli võõrutuse anksiogeensele toimele pluss-puuri testis. Nende andmete põhjal võib väita et NOergilised ülekandeteed ei oma olulist rolli etanooli võõrutussündroomi arengus.
4. Etanooli ja L-NOARGi koosmanustamine põhjustas olulise toksilisuse, mis väljendus suurenenud letaalsuses ja 14 päeva jooksul esinenud kaalukaotuses. Selle efekti aluseks võib olla etanooli organeid kahjustava toime tugevdamine.
5. NOS inhibiitoreid on kirjanduses pakutud välja võimaliku ravimina etanooli võõrutussündroomi ja etanoolist põhjustatud organite kahjustuse korra. Kuid meie andmete põhjal on võimalikud tõsised farmakodünaamilised ja/või farmakokineetilised interaktsioonid etanooli ja NOS inhibiitorite vahel.

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Teadustöö põhisuunad: Kroonilise alkoholismi tagajärgede uurimine — rakusurma tekkepõhjused (nekroos, apoptoos), kroonilise alkoholismi markeri väljatöötamine raskes alkholijoobes inimestel, CDT (Carbohydrate-Deficient-Transferrin) määramine.

Eesti Kohtuarstide Seltsi liige

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