INACTIVATION OF CEREBELLAR NITRIC OXIDE SYNTHASE BY ETHANOL IN VITRO

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Abstract — The N-methyl-D-aspartate receptor/nitric oxide synthase (NOS)/guanylate cyclase pathway, which plays a crucial role in synaptic plasticity in the brain, is modulated by ethanol. We studied the effect of ethanol in vitro on NOS in rat cerebellum and showed that ethanol (25-200 mM) inactivated NOS in a dose-dependent manner. This inactivation was prevented by the biopterin cofactor tetrahydrobiopterin (BH4) as well as by L-arginine, a NOS substrate, but not by NADPH. These results suggest that ethanol reduces NOS activity by modulating the conformation of the enzyme and thereby its stability, probably by interacting with the binding sites of BH4 and/or of L-arginine. Our data also suggest that inactivation of NOS may contribute to the decrease in the cGMP level, and thus may play a role in the pharmacological actions of ethanol in vivo.

INTRODUCTION

Nitric oxide (NO) is involved in several physiological and pathological processes in mammalian systems. Besides its role as endothelial-derived relaxing factor (Palmer et al., 1988) or as a mediator of macrophage cytotoxicity (Hibbs et al., 1988; Stuehr et al., 1989), NO appears to be an important cellular messenger in the brain. The N-methyl-D-aspartate (NMDA) receptor, a member of the family of receptors mediating the action of glutamate, when activated, becomes permeable to Ca\(^{2+}\) and this induces NO production and cGMP synthesis. NMDA receptors and the NO/cGMP system appear to play a crucial role in the regulation of synaptic transmission in the brain.

It is well-known that the cerebellum is disturbed by alcohol exposure during development and adulthood. Alcohol-induced disturbances are expressed as changes in learning, memory and vision (Sullivan et al., 1995). The glutamate receptor/nitric oxide synthase (NOS)/guanylate cyclase pathway, which plays a role in synaptic plasticity (Boxall and Garthwaite, 1996), is modulated by ethanol. It is well-documented that ethanol reduces the potency of glycine to enhance the response of NMDA receptors to glutamate and decreases thereby Ca\(^{2+}\) influx and cGMP production (Hoffman et al., 1989). In vivo, ethanol has been demonstrated to lower cGMP in the brain, with a particularly pronounced effect in the cerebellum. The fall in cGMP level may be linked to multiple factors including a decrease in NOS activity. Studies on brain indicate that ethanol can influence NO synthesis. In fact, Fitzgerald et al. (1995) have shown that chronic alcoholization (3 months) diminished significantly the neuronal nNOS (nNOS) level in some brain regions (e.g. hippocampus, striatum) whereas there was an increase of nNOS level in nucleus accumbens. In an in vitro study, Brien et al. (1995) showed that ethanol exposure did not modify NOS activity in hippocampus or cerebellum.

*NO is synthesized from L-arginine with L-citrulline as a co-product by various isoforms of NOS that differ in subcellular localization,
regulation and functional roles. The activity of NOS depends on NADPH, molecular oxygen, FAD, FMN, haem and (6R) tetrahydrobiopterin (BH4). NOS molecules appear to have fairly high affinity for BH4, since nanomolar concentrations were found to be sufficient for half-maximal stimulation of the different isoforms. Although the requirement for BH4 for maximal catalytic activity is well established, the mechanism by which BH4 stimulates NOS activity remains unclear. This compound, a well-known cofactor of monooxygenases for aromatic amino acid hydroxylation, was found to stimulate the cytokine-inducible, Ca2+-independent NOS isoform expressed in macrophages and glial cells (Tayeh and Marietta, 1989; Simmons and Murphy, 1992) as well as the Ca2+-calmodulin dependent constitutive isoform expressed in endothelium and neurons (nNOS) (Mayer et al., 1990; Giovanelli et al., 1991; Werner-Felmayer et al., 1993). Current experimental evidence hints at a dual mode of action of BH4 involving also participation in NO synthesis, i.e. redox cycles and an allosteric effect on the NOS protein (Tayeh and Marletta, 1989; Giovanelli et al., 1991; Baeck et al., 1993; Mayer and Werner, 1995).

This study was undertaken to determine the influence of ethanol in vitro on the constitutive isoform of NOS expressed in the cerebellum, where nNOS is particularly abundant. We also studied the effects of L-arginine and of BH4 on ethanol-mediated NOS inhibition.

MATERIALS AND METHODS

Chemicals

L-[3H]arginine (63 Ci/mmol; 1 Ci = 37 GBq) and cGMP radioimmunoassay kit were purchased from Amersham, Life Science (Les Ulis, France). NADPH was purchased from Boehringer Mannheim (Meylan, France). Chromatography columns (Dowex AG5OW-X8) were purchased from Biorad (Ivry sur Seine, France). All other chemicals were purchased from Sigma Chemicals (Paris, France).

Animals

Male Sprague–Dawley rats (average weight 180 g) were used. They were maintained on a standard laboratory diet (Iffa-Credo, UAR). Overnight fasted rats were used for all the experiments.

Assay of NOS activity

Preparation of cerebellar homogenates. The rats were killed by decapitation and the cerebellum was quickly removed, placed on an ice-cooled plate, cleaned of adhering blood and homogenized with a Teflon pestle in ice-cold 20 mM Tris–HCl (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged sequentially: at 2000 g for 10 min at 4°C, then at 15 600 g for 10 min at 4°C and finally at 100 000 g for 1 h at 4°C. The cytosolic fraction was passed through cation-exchange chromatography columns (Dowex AG5OW-X8, Na+ form) equilibrated with 20 mM Tris–HCl (pH 7.2) containing 1 mM EDTA to remove endogenous L-arginine.

Assay of NOS activity. NOS activity was assayed by measuring the conversion of L-[3H]arginine to L-[3H]citrulline as described previously (Bredt and Snyder, 1990) with modifications. The cytosolic fraction (100–200 μg of protein) was incubated at 37°C with 1 mM NADPH, 50 μM L-arginine, 2 μCi/ml L-[3H]arginine and 2.5 mM CaCl2 in a total volume of 230 μl. Reactions were stopped after 10 min by adding 2 ml of ice-cold 20 mM HEPES (pH 7.2) containing 2 mM EDTA to 170 μl of reaction mixture. Samples were applied to chromatography columns (Dowex AG5OW-X8, Na+ form) equilibrated with 20 mM HEPES (pH 5.5) and the L-[3H]citrulline newly synthesized was specifically eluted with 2 ml of distilled water. Liquid scintillation fluid (emulsifier Safe Packard) was added and L-[3H]citrulline was quantified in a Beckman spectrophotometer. NOS activity was expressed as pmol of citrulline formed per mg of protein per min.

Non-catalytic inactivation. Cytosolic fraction (100–200 μg of protein) was preincubated in the absence or the presence of various ethanol concentrations (25–200 mM) at 37°C in 20 mM HEPES (pH 7.2) containing 0.32 M sucrose, 1 mM EDTA and 9 μg/ml of calmodulin in a total volume of 170 μl for 10 min before NOS catalytic determination. Such preincubation was carried out in the absence of L-arginine, i.e. when NOS is catalytically inactive. Samples were assayed immediately for NOS activity. Nitro-L-arginine methyl ester (1 mM) was added to blank samples.
Measurement of cGMP level

Ethanol, as a 20% (v/v) solution, was administered intraperitoneally to treated rats (50 mmol/kg body wt) and control rats were injected with the same volume of saline solution 4 h before death by decapitation. The cerebellum was quickly removed, placed on an iced plate, cleaned of adhering blood and homogenized with a Teflon pestle in ice-cold 50 mM Tris–HCl (pH 7.5) containing 4 mM EDTA. The homogenate was centrifuged at 12,000 g for 2 min, the supernatant was boiled for 3 min at 100°C and then centrifuged at 12,000 g for 2 min. The deproteinized supernatant was used for the measurement of cGMP levels using a commercially available radioimmunoassay kit (Amersham). cGMP level was calculated in terms of pmol per g of cerebellum.

Protein determination

Protein concentrations were determined according to Bradford (1976) with bovine serum albumin as standard.

Statistical analysis

Results are expressed as means ± SEM and statistical significance was analysed with Student’s t-test in all experiments, except when stated otherwise.

RESULTS

Inactivation of nNOS by ethanol

We first determined whether ethanol in vitro promoted a non-catalytic inhibition of NOS activity. For this purpose, cerebellar cytosolic fractions were either preincubated at 37°C or pre-exposed at 0°C, in the presence of 200 mM ethanol, for 10 min before catalytic NOS activity determination. We used a high concentration of ethanol (200 mM) to screen for the ethanol’s effect on NOS. As shown in Fig. 1, ethanol significantly (P < 0.001) decreased NOS activity only when cytosolic fractions had been preincubated at 37°C, suggesting that ethanol produced a non-catalytic inactivation of NOS. Interestingly, we noticed that preincubation of cytosolic fractions at 37°C in the absence of ethanol decreased nNOS activity. In quite similar conditions, inactivation of purified porcine cerebellum NOS during preincubation has also been reported (Hofmann and Schmidt, 1995). We studied the effect of various ethanol concentrations on the cerebellar NOS and showed that ethanol from 25 to 200 mM inhibited the formation of L-[3H]citrulline from L-[3H]arginine in a dose-dependent manner (Fig. 2). The half-maximal effective concentration was 177 mM. The inhibition of nNOS by ethanol was already statistically significant for concentration of ethanol as low as 25 mM (10% inhibition, P < 0.02).

Modulation of ethanol-induced NOS inactivation by some cofactors

The effects of L-arginine, BH4 and NADPH on NOS activity were determined. The compounds were added to cerebellar homogenates during the preincubation period at 37°C, in the absence or presence of ethanol (Fig. 3). The inactivation of NOS by preincubation at 37°C in the absence of ethanol was almost fully overcome by 5 μM L-arginine or 50 μM BH4 (control 0°C: 244.3 ± 31.6 pmol/mg/min; control 37°C: 112.4 ± 40.8; 5 μM L-arginine: 223.9 ± 59.1; 50 μM BH4: 197.3 ± 27.1), whereas 1 mM NADPH was ineffective. In the presence of
IC 50 = 177 mM

Fig. 2. Inactivation of nNOS as a function of ethanol concentration.
Cerebellar cytosolic fractions were preincubated at 37°C in the presence of ethanol at the indicated final concentrations for 10 min before catalytic determination. Values are means ± SEM of five independent determinations; each was performed in duplicate. Statistical significance was analysed with Student’s paired t-test. **P < 0.02, ***P < 0.01, +++P < 0.001, all compared with control.

200 mM ethanol, the drop in NOS activity was also offset by 5 µM L-arginine or by 50 µM BH4 whereas 1 mM NADPH was still ineffective.

These results are consistent with the proposal that optimal concentrations of L-arginine and BH4 stabilized the NOS protein, allowed it to be converted into a catalytically active homodimer and could prevent the loss of NOS activity. The inactivation of NOS in the presence of ethanol should result from the interaction of ethanol itself or its by-products with some NOS sites which are no longer accessible when L-arginine or BH4 are at optimal concentrations.

Because of the proposed reducing role of BH4, we tested a general reducing agent, ascorbate (2 mM) added for the preincubation period, and observed that ethanol maintained its inhibitory effect on NOS activity (data not shown). This result is similar to that obtained with the 1 mM concentration of another reductant, NADPH (Fig. 3).

It was interesting to observe that no protective effect against ethanol-induced NOS inactivation was observed when BH4 was added after the preincubation period (Fig. 4).

Interaction between BH4 and ethanol in the modulation of NOS activity

To investigate the mechanism accounting for inactivation of brain NOS by ethanol, increased concentrations of BH4 (from 2.5 to 500 nM) were added during the preincubation period in the presence or in the absence of ethanol (200 mM) and the conversion of L-[3H]arginine to L-[3H]citrulline was measured (Fig. 5). BH4 protected NOS against ethanol-induced inhibition in a dose-dependent manner. At a saturating concentration of BH4 (500 nM), the activities obtained in the absence or in the presence of ethanol were quite similar (315.3 ± 34.9 and 300.9 ± 38.3 pmol/mg/min). Moreover, the apparent affinity
of NOS for BH$_4$ was decreased by ethanol since the $S_{0.5}$ values for BH$_4$ were 17.2 nM in control and 33.6 nM in the presence of ethanol (Fig. 5).

These results suggest that ethanol inhibited NOS in a competitive manner with respect to the biopterin cofactor.

**cGMP level**

At 4 h after an acute ethanol administration, the cGMP level in the cerebellum was significantly decreased from 47.9 ± 3.6 to 37.9 ± 3.2 pmol/g of cerebellum ($P < 0.001$).

**DISCUSSION**

The present data demonstrate that NOS is sensitive to inactivation by ethanol. The inactivation of cerebellar NOS occurred at concentrations of ethanol that are relevant to human ethanol consumption (115 mg/dl or 25 mM).

The ethanol-mediated inactivation of NOS was prevented by BH$_4$ and L-arginine. BH$_4$ may serve to keep reduced a catalytic component of NOS and possibly prevents the inhibitory action of ethanol by this mechanism. Nevertheless, the protective effect of BH$_4$ does not appear to be a general phenomenon of reducing agents, since ascorbate and NADPH were unable to offer any protection. However it cannot be excluded that BH$_4$ acts as a specific reducing agent, in the core of the enzyme.

BH$_4$, as well as L-arginine, may also protect NOS against ethanol-induced inhibition by stabilizing the enzyme. In fact, Klatt et al. (1994) showed that both L-arginine and BH$_4$ induced changes in the conformation of nNOS, resulting in enhanced affinities of the two binding domains for their respective ligands. This positive co-operation between the substrate and pteridine binding domains points to an allosteric interaction of the two sites. The above authors suggested that both L-
arginine and BH₄ played a very important role in the stability of the enzyme and confirmed this result by demonstrating that L-arginine and BH₄ synergistically converted neuronal NOS into an exceptionally stable homodimer that survived in denaturing agents such as SDS 2% and 2-mercaptoethanol 5% (Klatt et al., 1995).

As we showed that inactivation of NOS by ethanol was modulated by BH₄, it can be suggested that ethanol induced a loss in NOS activity by altering the binding sites of biopterin and/or of the substrate, either by inducing the monomerization or by destabilizing the dimeric structure. It thus appears that the inhibition of nNOS by ethanol may be related to the conformation of the enzyme.

In quite similar conditions to those presently used to demonstrate the protective effect of BH₄ on NOS inactivation, Hofmann and Schmidt (1995) have shown that the maintenance of the reduced state of protein thiols by adding GSH during the non-catalytic preincubation resulted in a stabilized NOS. Brien et al. (1995) reported that ethanol exposure in vitro (25–100 mM) did not affect NOS activity. However, the experimental conditions that we used were quite different. In fact, we used cerebellar homogenates more diluted and submitted to dialysis to remove endogenous L-arginine. According to the results presented here, an ethanol inhibitory effect was evident only when L-arginine or BH₄ was either absent or at suboptimal concentrations. Therefore, the differences in experimental conditions could explain the apparent discrepancy.

The present results agree well with a recent study (Matsubara et al., 1996) reporting that tryptophan hydroxylase, an enzyme requiring BH₄ as a cofactor, is inhibited by ethanol in a
ETHANOL INACTIVATES NOS

Fig. 5. Protective effect of various concentrations of BH4 on NOS inactivation by ethanol.

Cerebellar cytosolic fractions were preincubated at 37°C in the absence (control) or the presence of ethanol (200 mM), with BH4 to give the indicated final concentrations (2.5–500 nM), for 10 min before catalytic determination. Values are means ± SEM of three independent experiments, each was performed in duplicate. n.s.: *P > 0.05, **P < 0.01 both in comparison with respective controls.

non-competitive manner with respect to both the bioppterin cofactor and the substrate.

In the light of the diverse pharmacological actions of ethanol, it is possible that some effects of ethanol may be due to a decrease in NOS activity. In view of our results, it appears that the magnitude of ethanol-induced inactivation depends on the availability of substrates and co-factors. Recent studies indicate that L-arginine is found primarily in astrocytes in vivo, suggesting that glia may function as a cellular store for the NO production in neurons (Aoki et al., 1991). Some impairment could occur in the supply of L-arginine into neurons during alcoholization as well as in BH4 metabolism. This latter alteration has been reported in some neurodegenerative diseases and ageing (Levine, 1988; Duch and Smith, 1991).

Our data, as well as prior studies (Volicer and Klosowicz, 1979; Ferko et al., 1982), show that intoxicating doses of ethanol lower cerebellar cGMP levels in vivo. This fall in cGMP level reflects functional changes in the NMDA receptor/NOS/guanylate cyclase pathway and may be linked to multiple factors, including the inhibition of the receptors as well as the inhibition of NOS as
suggested by the present data.

In summary, the results of the present study demonstrate that ethanol reduces NOS activity probably by interacting with the binding sites of BH4 and/or of L-arginine. This inactivation of NOS may be a contributing factor in the pharmacological effects of ethanol on the central nervous system.

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