A Comparison between Acute Exposures to Ethanol and Acetaldehyde on Neurotoxicity, Nitric Oxide Production and NMDA-Induced Excitotoxicity in Primary Cultures of Cortical Neurons

Jiz-Yuh Wang[1,2], Ju-Yu Wang[1,4], Yung Wang[3] and Jia-Yi Wang[1,2]

[1]*Graduate Institute of Life Sciences*
Departments of [2]*Physiology and* [3]*Pharmacology*
National Defense Medical Center
Taipei 114, Taiwan, ROC
and
[4]*Hung-Kuang Institute of Technology*
Taichung 433, Taiwan, ROC

Abstract

Chronic exposure of primary neuronal cultures to ethanol has been shown to potentiate N-methyl-D-aspartate (NMDA) receptor-mediated processes, such as nitric oxide (NO) formation and excitotoxicity. In the present study, we compared the effects of acute ethanol and acetaldehyde on NMDA receptor-mediated excitotoxicity and NO production in primary cultures of rat cortical neurons. The delayed cell death induced by NMDA (300 mM, 25 min) was evaluated by morphological examination and by measuring the release of the cytotoxic indicator, lactate dehydrogenase, in the culture media 24 hours after the NMDA exposure. The accumulation of nitrite, as an index of NO production, was also measured 24 hours after NMDA treatment. NMDA caused a dose-dependent cell death and nitrite accumulation, both effects were blocked by pretreatment of MK-801 (100 μM). Acute exposure to ethanol (1-1000 mM) or acetaldehyde (0.1-1 mM) for 35 minutes did not affect neuronal viability in the following 24-hr period. However, acute exposure to acetaldehyde (≥10 mM) was neurotoxic. Neither ethanol nor acetaldehyde changed basal nitrite levels in the culture media. Acute ethanol (50-400 mM, 10 min) given before the NMDA treatment (25 min) resulted in a concentration-dependent suppression of the delayed cell death. The NMDA-induced NO production was, however, not affected by ethanol. Neither the NMDA excitotoxicity nor NO production was affected by acute ethanol given after NMDA treatment. Acute acetaldehyde (0.01-0.5mM, 10 min) given before or after NMDA treatment had no effect on delayed NMDA neurotoxicity and NO production. Our data suggest that acute exposure to ethanol is not neurotoxic and is even protective against delayed NMDA-excitotoxicity when given before but not after NMDA treatment. Neither NO nor metabolism of ethanol to acetaldehyde is required for ethanol-mediated suppression of NMDA excititoxicity. Acetaldehyde, on the other hand, is toxic by itself at low concentrations (≥10 mM). Furthermore, acute exposure to non-toxic concentrations of acetaldehyde could not protect cortical neurons against NMDA-induced excitotoxicity.

Key Words: cortical neurons, ethanol, acetaldehyde, NMDA, nitric oxide, neurotoxicity.

Introduction

Ethanol-induced brain damage occurs not only with in utero exposure but also with adult alcohol abuse. Despite a large amount of research, the mechanism(s) underlying the neurotoxicity of ethanol remain unknown. It is well-known that excessive release of glutamate resulted from the brain injury

Corresponding author: Dr. Jia-Yi Wang, Department of Physiology, National Defense Medical Center, 161, Sec.6, Ming-Chuan E. Rd. Taipei 114, Taiwan, ROC. Tel: 886-2-8792-3100 ext.18607, Fax: 886-2-8792-4893. E-mall: jywang@ndmctsgh.edu.tw
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triggers a sequence of events that result in neuronal degeneration and eventually death. Although activation of other glutamate receptors may also contribute to glutamate excitotoxicity, it has been indicated that activation of N-methyl-D-aspartate (NMDA) receptors is the most important neurotoxic processes leading to neuronal death (3, 4, 27, 32). It is well-known that excessive release of glutamate resulted from the brain injury leads to the stimulation of NMDA receptors and elevates the free intracellular Ca^{2+} levels, which leads to activation of Ca^{2+}-dependent NOS and the synthesis of NO (6).

Recent evidence indicates that ethanol may affect brain injury by interacting with glutamate receptor, especially NMDA receptor, and represent important sites of actions of ethanol in the brain. Chronic exposure of primary neuronal cultures to ethanol has been shown to potentiate NMDA-mediated processes, such as nitric oxide (NO) formation and excitotoxicity (2, 5, 34). Acute exposure to ethanol, however, attenuates NMDA-induced neuronal injury in neuronal cultures (2). Electrophysiological studies have shown that NMDA currents expressed in Xenopus oocytes are inhibited by pharmacologically relevant concentrations of ethanol (8). It has been shown that ethanol inhibits NMDA-induced ion currents (23, 25, 29, 40); Ca^{2+} influx (17, 31), elevation of intracellular Ca^{2+} levels (7, 9), cGMP production (17), and neurotransmitter release (10, 14). However, whether ethanol reduces NMDA-mediated NO production remains unknown. Despite the evidence indicating that NO mediates glutamate excitotoxicity *in vivo* and *in vitro*, the issue regarding whether NO is neurotoxic or neuroprotective is still a matter of debate (20). NO may have a "double-edged" role, i.e. neuroprotection and neurotoxicity, depending on the cellular source of NO and its targets, the amount of NO being produced, and redox state of the tissue (41, 42).

Several toxic actions implicated in ethanol abuse in human is partially exerted by acetaldehyde (39). It has also been shown that a catalase-mediated oxidation of ethanol is present in rat brain homogenates (1, 43, 44), suggesting that acetaldehyde is produced directly in the brain and that it may be the agent mediating some of the effects of ethanol. Since the effect of acetaldehyde on NMDA-mediated responses has not been reported, it is of interest to examine the effect of acetaldehyde by itself, and its effects on NMDA-induced neurotoxicity and NO production in neurons as compared with ethanol.

Meterials and Methods

Cortical Neuronal Culture

Primary cortical neuronal cultures were prepared from fetal Sprague-Dawley rats [embryonic day 16-17 (E16-17)] as previously described (38). In brief, whole cerebral hemispheres were collected aseptically in Ca^{2+}/Mg^{2+} free Hank's Balanced Salt Solution (HBSS; Gibco-BRL, Gaithersburg, MD). The blood vessels and pia matter were removed carefully. Brain cortical tissues were pelleted by centrifugation at 500×g for 10 min. Pellets were dissociated by gentle trituration in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (10% FBS/DMEM). The cell suspension diluted in additional 10% FBS/DMEM medium is plated at a density of 1×10^6 cells/ml (well) in 12-well (22 mm) cultured plates that has been previously coated with poly-L-lysine. Cultures were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air. The cytosine arabinoside (ARC, the final concentration is 5 μM) was added 60-72 hours after plating and remained for 48 hr to inhibit non-neuronal cell division. Two days later, ARC containing medium were replaced by 10% FBS/DMEM medium. Subsequent media replacement was carried out three days later with 5% FBS/DMEM for another 2-4 days. Experiments were performed on cultures at 10-12 days after plating. All cultures were examined microscopically before being used.

Immunocytochemical Staining

Neurons were stained with an antibody against microtubule-associated protein-2 (MAP-2), a stringent marker for both the cell body and neurites. The underlying glial cells in the same well or in sister wells were stained by antibody against glial fibrillary acidic protein (GFAP), the marker protein for astrocytes. Cultures were washed with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature, and then incubated with primary antiserum against MAP-2 (Pharmingen, San Diego, CA) or GFAP (Chemicon Inc. Temecula, CA, U.S.A.). For visualization, a LSAB-universal kit (Dako) or a standard avidin-biotin peroxidase-diaminobenzidine technique (a biotinated secondary antibody plus Vectastain ABC kit from Vector Lab. Inc. Burlingame, CA) was used. Our neuronal cultures consisted of approximately 90% neurons and 10% astroglia as we previously described (38).

Exposure to Ethanol or Acetaldehyde with or Without NMDA Treatment

The procedure for examining NMDA-mediated excitotoxicity was based on a method described by Choi (4). The serum containing DMEM was aspirated from the culture plate and the cells were washed with Mg^{2+}-free HEPES-bicarbonate (HB) buffer (25 mM

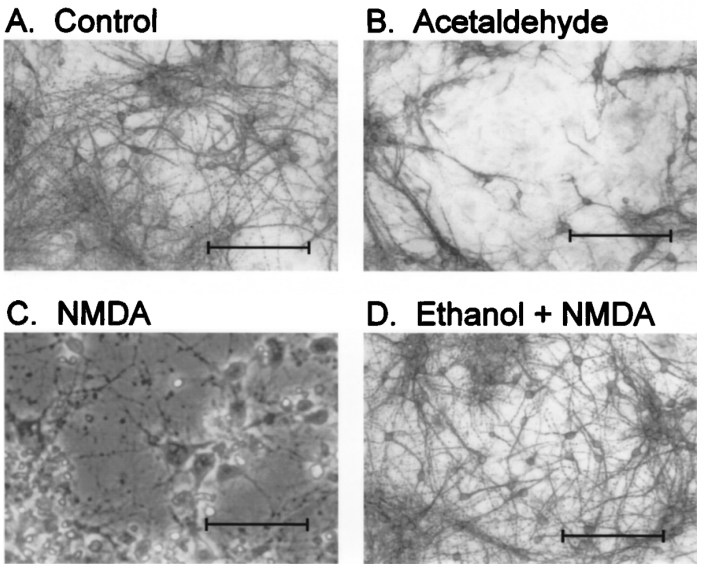

Fig 1. Brightfield photomicrograph of primary cortical neuronal culture stained with antibody against MAP-2. Clusters of MAP-2 positive neurons exhibited extensive neuritic networks in control cultures (A). Acute exposure of the cultures to acetaldehyde (10 mM, 35 min) resulted in the loss of neuronal cell bodies and their neurites (B). Extensive disintegration of neuronal processes and swollen (edematous) or granule cell bodies were observed in cultures 24 hr following the treatment with NMDA (300 mM, 25 min) (C). Pretreatment of ethanol (200 mM) prevented cells from NMDA excitotoxicity so that no significant morphological differences from control cells were observed (D). Bar = 100 μM

HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, and 15 mM glucose; pH 7.4) and incubated in HB buffer with or without NMDA for 25 min unless otherwise noted. For ethanol and acetaldehyde experiments, some cultures were exposed to ethanol or acetaldehyde without NMDA for 35 min, and the other cultures were for 10 min prior to or after the addition of NMDA and incubated for another 25 min with NMDA. Such exposure paradigm was based on a previously described method (2). Exposures to ethanol or acetaldehyde with or without NMDA were terminated by rapid aspiration of the HB buffer followed by washing the cells with serum-free MEM. The culture plates containing serum-free MEM were returned to the incubator for further 24 hr unless otherwise noted.

Analysis of Cytotoxicity and Viability

The morphology of cell bodies and neurites was assessed qualitatively by MAP-2 immunostaining and microscopic examination. In experiments examining NMDA injury, phase-contrast microscopy was supplemented with trypan blue staining (0.4% for 5 minutes). Following NMDA exposure for 25 min in the presence and absence of ethanol or acetaldehyde, the culture medium was replaced with serum-free MEM (without phenol red). Neuronal cell death was estimated by microscopic examination and quantified by measurement of LDH activity released into the bathing medium 24 hr following the NMDA exposure. A diagnostic kit (Sigma. St. Louis, MO, U.S.A.) was used to measure LDH activity in the medium of cell culture. Cytotoxicity was quantitatively assessed according to previously published methods (21, 36, 38). LDH activity (units/ml) was calculated from the slope of the decrease in optical density at 340 nm over 3-min time period. One unit of LDH activity is defined as the amount of enzyme that catalyzes the comsumption of 1 mmol of NADH per minutes.

Measurement of Nitrite Accumulation

Since NO itself was unstable, NO production was determined by the measurement of nitrite, a stable oxidation product of NO. Nitrite was measured in the media (serum-free and without phenol red) 24 hr following the experiments by a spectropho-tometric assay based on the Griess reaction (15). Griess reagent consists of 0.1% N-(1-naphthyl)ethylene diamide dihydrochloride, 1% sulfanilic acid, and 2.5% phosphoric acid. Duplicate aliquots (100 μl) of culture medium were removed and mixed with an equal volume of Griess reagent. After 30-min reaction, the absorbance was measured at 540 nm. Sodium nitrite in assay medium in a range between 0.5 and 32 μM was used to prepare a standard curve for quantitation.

Results

Effect of Acute Ethanol or Acetaldehyde on Cell Viability and NO Production

Immunocytochemical analysis with an antibody

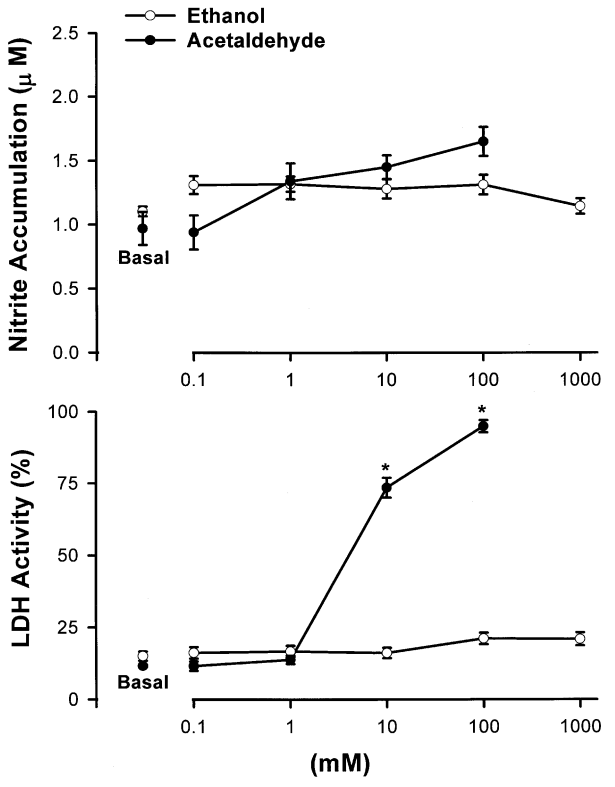

Fig. 2. Concentration-response of ethanol or acetaldehyde exposure in cortical neuronal cultures. Neuronal cultures were exposed to various concentrations of ethanol or acetaldehyde for 35 min. Nitrite accumulation (TOP) and LDH activity (BOTTON) were quantitated by measuring media 24 hr later following ethanol or acetaldehyde washout. Values of LDH activity are scaled to the mean value measured after exposure of sister cultures to 300 μM NMDA for 24 hr (=100% LDH activity). Each data point represents the mean±SEM of 7-9 samples performed in duplicates. Data were analyzed for significant difference by one-way ANOVA, followed by Newman-Keuls' test. (* indicates significant difference from the corresponding control value: $p<0.05$)

against MAP-2 revealed healthy MAP-2 positive neurons with extensive neurites in the control cultures (Fig. 1, A). The soma as well as the extensive neuritic networks could be stained by MAP-2 antibody (Fig. 1, A). Acute exposure of neuronal cultures to ethanol (0.1~1000 mM) or acetaldehyde (0.1~1 mM) for a 35-min period was not toxic as the morphology of MAP-2 positive neurons in treated cultures was indistinguishable from control cultures (data not shown). However, acute exposure of the cultures with 10 mM acetaldehyde for 35 min resulted in damage to the MAP-2 positive neurons which was characterized as the loss of neuronal cell bodies and their neurites (Fig. 1, B).

Ethanol by itself at concentrations 0.1~1000 mM exerted no influence on nitrite accumulation and LDH activities in neuronal cultures (Fig. 2). The same results were obtained in nitrite accumulation and LDH activity from cultures treated with acetaldehyde at concentration from 0.1 mM to 1 mM. Acetaldehyde, on the other hand, was toxic by itself at low concentrations (≥10 mM).

NMDA-induced Delayed Neurotoxicities and NO Production

NMDA-induced neuronal cell injury was assessed by phase-contrast microscopy at various times up to 24 hr following NMDA exposure. In contrast to sham-treated sister cultures (Control, Fig. 1A), disruption of neuronal cell bodies and the neuritic network was gradually evident (Fig.1 C). The nitrite production and the LDH release gradually increased after a short (25 min) exposure to NMDA, indicating a delayed type of neuronal injury. Figure 3 shows the nitrite accumulation and LDH activity at various times up to 24 hr after a short exposure to NMDA (300 μM). In order to clearly represent the degree of cell injury, values of LDH activity are scaled to the mean value measured after exposure of sister cultures to 300 μM NMDA for 24 hr (=100% LDH activity). Treatment of neuronal cultures with various concentrations of NMDA in the Mg^{2+}-free condition caused a concentration-dependent NO production and cell death, as reflected the increases in nitrite accumulation and LDH activity in the culture media at 24 hr after NMDA washout (Fig. 3). Exposure to NMDA (300 μM, 25 min) caused about 60%~70% neuronal death and nitrite accumulation in neuronal culture. The NMDA receptor antagonist MK-801 (100 μM) prevented the NMDA-induced increases in nitrite production and LDH release (Fig. 3, right), suggesting that the neurotoxic effects and NO production are indeed mediated via the NMDA receptor.

Effect of Acute Exposure to Ethanol or Acetaldehyde before or after NMDA Treatment

Pretreating the neuronal cultures with ethanol (50~400 mM) for 10 min resulted in a concentration-dependent inhibition of NMDA neurotoxicity, but ethanol exerted no effects on NMDA receptor-mediated NO production (Fig. 4, left). However, ethanol treatment following NMDA exposure could not attenuate the NO production and cell injury (Fig. 4, left). In contrast to ethanol, neither pre-treatment or post-treatment of acetaldehyde exerted any significant effects on nitrite accumulation and LDH activity, even at concentrations up to 0.5 mM (Fig. 4, right).

Examination of neuronal cultures under phase-contrast microscope during the time of elevated LDH activity resulted from NMDA (300 μM, 25 min)

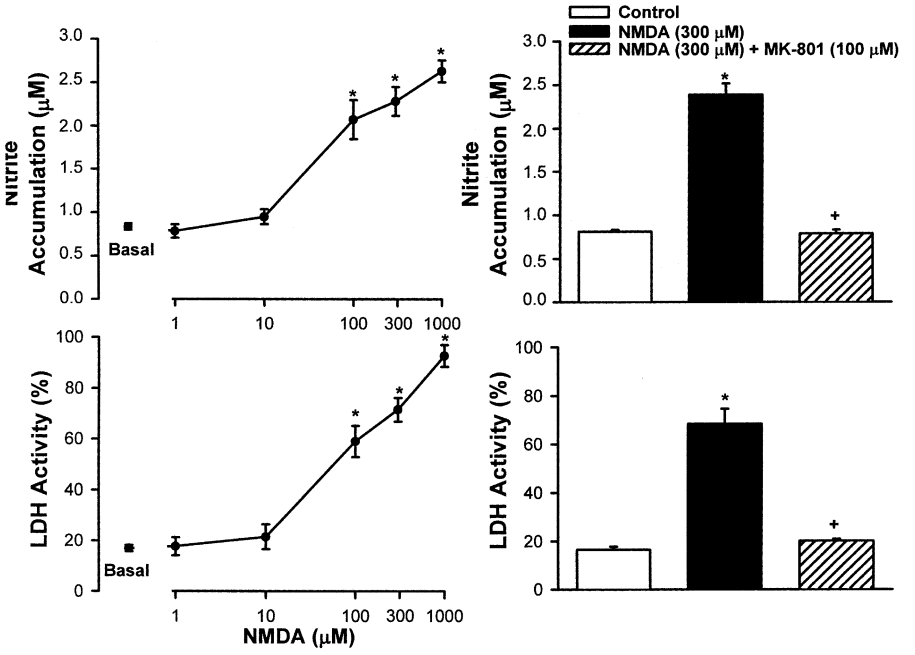

Fig. 3. (LEFT) Concentration-response for NMDA-mediated neurotoxicity and NO production in cortical neuronal cultures. NMDA was given in various concentrations to neuronal cultures for 25 min and then washed out. Nitrite accumulation (TOP) and LDH activity (BOTTOM) were measured 24 hr later after NMDA washout. (RIGHT) Neuronal cultures were preincubated with MK-801 (100 μM) for 10 min prior to the addition of NMDA (300 μM) for another 25 min. Each point represents the mean±SEM of 8-12 samples performed in duplicates. Values of LDH activity are scaled to the mean value measured after exposure of sister cultures to 300 μM NMDA for 24 hr (=100% LDH activity). Data were analyzed for significant difference by one-way ANOVA, followed by Newman-Keuls' test. (* indicates significant difference from the corresponding control value: $p<0.05$; + indicates significant difference from NMDA (300 μM) treatment group: $p<0.05$)

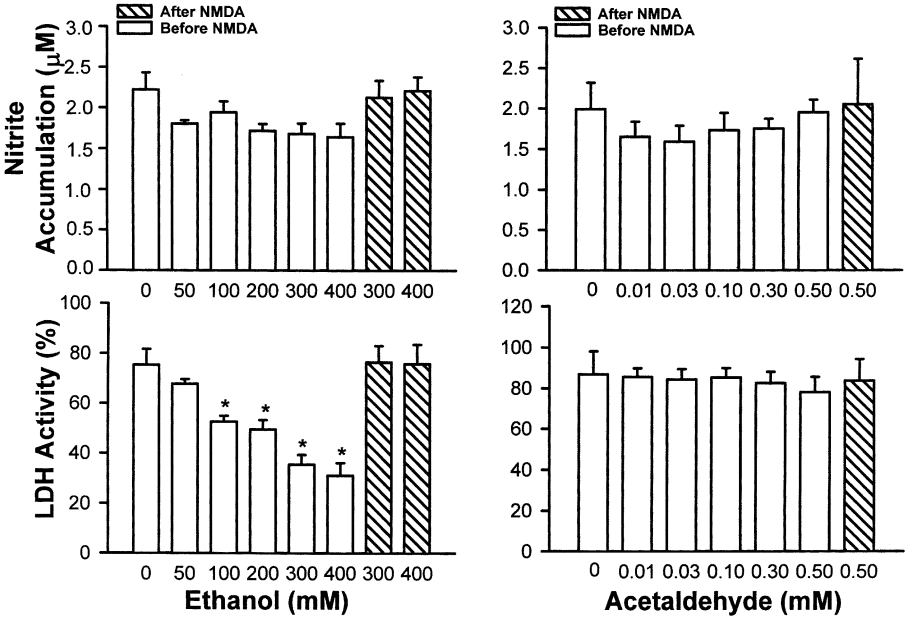

Fig. 4. Effects of ethanol (LEFT) and acetaldehyde (RIGHT) on NMDA-mediated NO production and neurotoxicity in cortical neuronal cultures. Neuronal cultures were incubated with the various concentrations of ethanol or acetaldehyde for 10 min either before or after the addition of NMDA (300 μM, 25 min) and then washed out. Nitrite accumulation (TOP) and LDH activity (BOTTOM) were measured 24 hr later following NMDA and ethanol / acetaldehyde washout. Data represent the mean±SEM of 8-12 samples performed in duplicates. Values of LDH activity are scaled to the mean value measured after exposure of sister cultures to 300 μM NMDA for 24 hr (=100% LDH activity). Data were analyzed for significant difference by one-way ANOVA, followed by Newman-Keuls' test. (* indicates significant difference from the NMDA alone (300 μM) treatment group, $p<0.05$)

showed obvious signs of neuronal injury as evidenced by extensive disintegration of neuronal processes and swollen (edematous)or granule cell bodies (Fig. 1C). However, when ethanol (200-400 mM) was given prior to and during 300 μM NMDA treatment, the protective effect ethanol against NMDA toxicities was found. The granulation and disintegration of cell bodies and nitritic processes were not obvious and there were no significant morphological differences in ethanol-pretreated and NMDA treated cells from control cells (Fig. 1D). Thus ethanol reduced NMDA-mediated neurotoxicity. These morphological observations are in agreement with LDH measurement.

Discussion

The results in the present study show that only acute exposure to ethanol, but not to its metabolite, acetaldehyde, inhibited NMDA-mediated excitotoxicity. However, neither ethanol nor acetaldehyde significantly reduced NO production during NMDA insults. The inhibition by ethanol of the NMDA-mediated neurotoxicity in cerebral cortical neurons in the present study is consistent with previous work showing that ethanol inhibits NMDA-induced excitotoxicity in neurons from whole brain regions (2). Our study focused on cortical neurons and found similar inhibitory effect. Our study further extend to examine the effects of acetaldehyde in primary cultures of cortical neurons on NMDA-induced excitotoxicity and NO production. Our result that the inhibitory effect of ethanol on NMDA excitotoxicity required the presence of ethanol prior to, but not after, NMDA treatment suggested that ethanol modulated NMDA-triggered cell death at an early stage of the cascade. Once the injury was initiated by NMDA, ethanol exerted little or weak effect.

The oxidative metabolism of ethanol by the cytochrome P450 2E1 (CYP2E1) has been recognized to contribute to the ethanol-induced deleterious effects through the induction of oxidative stress (28, 44). Metabolism of ethanol to acetaldehyde in the brain could be deleterious since it can react with cytoskeletal proteins, forming adducts (36). The oxidative metabolism of ethanol by the cytochrome P450 2E1 (CYP2E1) has been recognized to contribute to the ethanol-induced deleterious effects through the induction of oxidative stress (28, 33). Localization of CYP2E1 by fluorescence in situ hybridization demonstrated the constitutive expression of CYP2E1 preferentially in the neuronal cells in rat and human brain (36). Ethanol-derived acetaldehyde can also be produced primarily via catalase-mediated oxidation of ethanol in fetal or neonatal rat brain (12). Therefore, acetaldehyde production catalyzed by catalase may perhaps reflect the stages of ethanol oxidation in embryonic brain.

Our dose-response relationship of LDH activity for NMDA neurotoxicity is in close agreement with that previously described by Chandler et al. (2). NMDA receptor-mediated increase in LDH release showed a steep increase from minimal LDH release at 10 μM NMDA to a amount of LDH release at 100 μM NMDA may be indicative of a threshold level for neurotoxic receptor activation.

Neither ethanol nor acetaldehyde at concentrations examined were deleterious when given alone for a 35-min period. Although the ethanol concentration used in the present in vitro study is in the high sedative/hypnotic range for the nontolerant individual, tolerant alcoholic patients can achieve blood ethanol concentrations in this range. We have previously shown that concentrations of ethanol declined for an average of approximately 50 % after 24-hr exposure (38). With an estimated decline of ethanol concentration after 24 hr, the concentrations needed to produce the observable effects in the present study were not excessively high. This is consistent with the concentrations used in previous studies in which concentrations of ethanol (50-100 mM, 24-hr exposure) were also required to provide protection from NMDA excitotoxicity (IC_{50}=60 mM) (2, 26). Similar requirements for ethanol at these concentrations to achieve significant effects on peripheral blood vessels have also been reported. For example, acute alcohol exposure directly increases the NO production on pulmonary arteries *in vitro*. The minimal concentration of alcohol for such vasodilation is 0.04 % or 87 mM (16).

In order to examine the delayed toxic effects of NMDA in cortical neurons, we observed that exposure to NMDA for as little as 25 min was toxic to neurons in culture. LDH activity and NO production were not immediate but occured during post-24 hr period, indicating a delayed type of cell death. In various in vivo injury paradigms such as stroke or focal ischemia, delayed neuronal death is characteristic of excitotoxicity involving NMDA receptor stimulation. In our study this delayed neurotoxicity and NO production correlated with delayed morphological evidence of cellular damage (granularity and disintegration of neuronal processes) and nitrite accumulation in the medium. The morphological changes with NMDA toxicities was somehow different from those observed with acetaldehyde toxicities. In contrast to the extensive disintegration of neuronal processes and swollen (edematous) or granule cell bodies observed with NMDA treatment, the cell bodies of neurons treated with acetaldehyde became smaller and eventually disappeared without going through the edematous stage (Fig. 1C). This is consistent with recent studies demonstrating that the death mode of

acetaldehyde-induced cell death is apoptosis (18). In fact, the toxic mechanism of acetaldehyde in neurons is not well known. It has been shown that neuroactive amino acids content, choline acetyltransferase and acetylcholinesterase activities were all reduced remarkably by acetaldehyde treatment. Acetaldehyde may explain, at least in part, the alcohol-induced neurochemical alterations (22). However, acetaldehyde cytotoxicities have been found in cultured astrocytes. Acetaldehyde increases intracellular calcium levels, damages DNA and further activates apoptotic pathway (18).

Ethanol, but not acetaldehyde, inhibited NMDA receptor-mediated neurotoxicity at higher concentrations; however, ethanol exerted no effects on NO production. Electrophysiological and biochemical studies have shown that ethanol, at concentrations associated with mild to moderate intoxication, can reduce NMDA activated calcium currents and responses (13). Because excessive and/or inappropriate increases in intracellular calcium are thought to initiate neurotoxic processes during NMDA receptor stimulation, the protective action of ethanol may be due to reduced calcium influx through the NMDA receptor-coupled channel. Perhaps, NMDA receptor-mediated Ca^{2+} influx is not inhibited completely by ethanol, thus neuronal NOS can still be activated by lower levels of Ca^{2+} influxed from outside. It is possible that the small amount of calcium ions entered via NMDA receptor induces a large amount of calcium release from intracellular calcium stores such as mitochondria or endoplasmic reticulum. It is also possible that ethanol can directly interact with other calcium transport processes that may play a role in elevating intracellular calcium concentration, including up-regulation of voltage-sensitive calcium channels, and changes in Na^{2+}/Ca^{2+} exchange.

In the present study ethanol exerted no effects on NMDA receptor-mediated NO production in cortical neurons. Among brain cells, ethanol has inhibitory effects on NO production through iNOS in C6 glioma cell line (35) and primary culture of mixed glia (38). Even chronic ethanol exposure decreases hippocampal NOS activity (11) However, ethanol (50-200 mM) potentiated LPS-induced iNOS gene expression and NO formation in a rat BBB (endothelial) cell line (30). Ethanol does not affect norepinephrine-stimulated NO synthesis in medial basal hypothalamus (34). In our study, the inhibitory effect of ethanol on NMDA neurotoxicity is not due to the decrease in NO generation. Maybe NO is not the most critical molecule to damage cortical neurons. As to the effect of ethanol on NO production in vivo and in vitro, accumulating evidence has shown that nNOS activity in the various brain regions remains similar to the controls after both acute and chronic ethanol administration. Therefore, nNOS is resistant to ethanol at clinically relevant concentrations and that ethanol affects the NO-operated system in the brain through a pathway other than that of nNOS. (19). These observations perhaps imply that ethanol seems to selectively affect NO production by different brain cells, which may relate to reported behavioral interactions, but the extent and direction of change depends on cell type and length of exposure (34).

In summary, we conclude that acute ethanol by itself is not neurotoxic and is even protective against NMDA receptor-mediated neurotoxicity. The protective effect is not due to changing levels of NMDA-mediated NO production. Such effects are opposite to the effect of chronic ethanol exposure which always result in neurotoxicity. Acetaldehyde, on the other hand, is toxic by itself at low concentrations (≥10 mM). Furthermore, acute exposure to non-toxic concentrations of acetaldehyde could not protect cortical neurons against NMDA-induced excitotoxicity.

Acknowledgements

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