Alpha - Lipoic Acid Decreases DNA Damage and Oxidative Stress Induced by Alcohol in the Developing Hippocampus and Cerebellum of Rat

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Key Words

DNA damage • Fetal alcohol syndrome • Lipid peroxidation • Protein oxidation • Wistar rat

Abstract

Background / aims: The present study was initiated in order to investigate the protective effects of α -lipoic acid upon ethanol-induced DNA damage, lipid peroxidation and protein oxidation in the developing rat hippocampus and cerebellum. **Methods:** Pregnant Wistar rats received ethanol with, or without lipoic acid from gestation day (GD) 7 throughout lactation. The changes in DNA damage, protein carbonyl, lipid hydroperoxide, catalase and superoxide dismutase activities were measured in the hippocampus and cerebellum of male offspring at the end of the lactation period. **Results:** The results indicated that DNA damage, lipid peroxidation and protein oxidation in the hippocampus and cerebellum were significantly elevated in animals that received alcohol. However, the catalase and superoxide dismutase activity results showed patterns that differed from those of DNA damage, lipid peroxidation and protein oxidation. Lipoic acid treatment significantly decreased DNA damage compared with the group that were

administered alcohol alone, and restored the elevated protein carbonyl and lipid hydroperoxide levels to the levels of the control group. **Conclusions:** Our findings confirm that oxidative stress and DNA damage occur in the developing hippocampus and cerebellum as a result of alcohol administration, and also suggest that lipoic acid has protective effects as an antioxidant against alcohol-induced disorders in the developing hippocampus and cerebellum.

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Introduction

Maternal consumption of ethanol during pregnancy can injure the developing fetus, leading to dysmorphology and dysfunction in the offspring. Of the body systems that are adversely affected by prenatal ethanol exposure, the central nervous system (CNS) is considered to be the most sensitive to ethanol-induced perturbation. The term alcohol-related neurodevelopmental disorder (ARND) is used to describe the CNS dysfunction that may result from ethanol exposure during pregnancy [1].

The hippocampus is a selectively vulnerable structure to prenatal ethanol exposure [2]. It is generally accepted that prenatal ethanol exposure can alter hippocampal synaptic plasticity and can adversely affect hippocampal-dependent learning, and can also cause other mental defects [1]. The effects of ethanol upon the fetus are dependent on the gestational age during exposure [3], and the amount of ethanol to which the fetus is exposed [4]. The rat has been widely used as an animal model to study fetal alcohol syndrome (FAS) because of the similarities between the human and rat response to ethanol [5]. Rats respond to ethanol with neurotoxic effects that are dependent on the gestational stage at which the ethanol is administered [6]. During the second half of gestation in rats, in an interval that roughly corresponds to the second trimester in humans, ethanol exposure results in a decrease in neurons in both species [3]. Recently, several studies have examined the role of oxidative stress in developmental alcohol-mediated neurotoxicity, possibly via the formation of free radicals [7, 8]. Acute ethanol administration leads to lipid peroxidation, which is an indicator of oxidative stress [9, 10]. Increased oxidative stress is directly caused by ethanol and its oxidation products [10]. It has been demonstrated that ethanol induces the synthesis of cytochrome p450 (CYP2E1) in the brain. CYP2E1 is present in various brain regions, and may be an important source of ethanol-induced oxidative stress [11]. Alcohol exposure also induces oxidative damage to nucleic acids, as indicated by the increased levels of 8-hydroxyguanine (8-OHdG), an oxidatively altered base, in mouse and rat mitochondrial DNA [12, 13]. Finally, oxidative protein damage has been linked with alcohol exposure in a number of studies. Increased protein carbonyl formation, one of the most general and commonly used indicators of oxidative protein damage, has been observed in the blood, liver and intestinal mucosa following alcohol exposure [14-16]. Alpha lipoic acid (α LA) and its reduced form, dihydrolipoic acid (DHLA), have received attention as antioxidants with both preventive and therapeutic uses in humans and laboratory animals [17]. They have potential value during periods of oxidative stress. Both LA and DHLA also protect the integrity of cell membranes by interacting with antioxidant-glutathione (GSH) and vitamins E and C [17]. Lipoic acid is both water and fat-soluble, and may therefore regenerate water and fat-soluble antioxidants, such as vitamin E and vitamin C. LA and DHLA also eliminate hydroxyl radicals and hypochlorous acid with a potency comparable to glutathione and N-acetylcysteine [18].

The present study was designed to investigate the possible link between oxidative stress and DNA damage induced by ethanol exposure in the developing hippocampus and cerebellum of rats. The DNA damage induced by chronic ethanol administration was also studied.

Materials and Methods

Animals and treatments

All procedures on rats were performed according to the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985), as well as the specific rules of the "Animal Care and Use Committee", National Medical and Health Service. Adult female Wistar rats weighing 220 ± 20 g were bred with male breeders overnight, and were checked for the presence of the vaginal plug the next morning. The presence of a vaginal plug was considered to be indicative of conception, and that hour was designated as day 0 of gestation (GD0). On day GD7 of gestation, pregnant rats were randomly divided into three groups: 1) control, 2) ethanol and 3) ethanol-alpha lipoic acid groups. The control group received treatment only with a vehicle (normal sterile saline). Rats assigned to the alcohol group received 4.5 g/kg body weight ethanol (Merck- Germany) solution in saline (20% w/v) subcutaneously once a day. The ethanol-alpha lipoic acid group received 100 mg/kg lipoic acid (Sigma-Aldrich Inc) dissolved in 0.1mM NaOH intraperitoneal (i.p.) in addition to ethanol. The control and ethanol groups received an equal volume of Na OH i.p. The treatment was started on GD7 and continued through to 21 days postnatal (PN) (lactation period). Following birth, litters were culled to 3 or 4 male pups/mother to decrease possible nutritional deficiencies due to within litter competition. Due to important sex differences in the response to ethanol, we used only male pups for this study. After 35 days (14 days prenatal and 21 days postnatal) treatment of dams, 8 male pups from each group were anesthetized with ether.

Tissue preparation

The pups were killed by decapitation and the brain was immediately removed. The hippocampus and cerebellum were dissected for analysis of oxidative stress parameters and DNA damage. For the oxidative stress analyses, tissue samples were homogenized in 10mM Tris-HCl (pH 7.4), 5mM EDTA and centrifuged at $10000 \times g$ for 15 min at 4°C , and the supernatants were collected and stored at -80°C until measurement of requested parameters. Protein concentration was determined by the Bradford method, using bovine serum albumin as a standard.

Biochemical markers

Catalase activity in the supernatant was determined using a catalase assay kit (Cayman Chemical, MI, USA). This method is based upon the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2 . The formalde-

Fig. 1. Protein carbonyl levels in the hippocampus and cerebellum. Results shown are mean $\pm$ S.E.M. "a" significant at $p < 0.05$ with control. "b" significant at $p < 0.05$ with ethanol.

Fig. 2. LPO levels in the hippocampus and cerebellum. Results shown are mean $\pm$ S.E.M. "a" significant at $p < 0.05$ with control. "b" significant at $p < 0.05$ with ethanol.

hyde produced as a result of this reaction was measured spectrophotometrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole as the chromogen [19]. Catalase activity was expressed as nmol/mg protein/min. Tissue superoxide dismutase (SOD) activity was determined using a Cayman assay Kit (Cayman Chemical., MI, USA). This method uses tetrazolium salt to quantify the superoxide radicals generated by xanthine oxidase and hypoxanthine. The standard curve was generated using a quality-controlled SOD standard. Protein carbonyl content was measured in the supernatant using a protein carbonyl assay kit (Cayman Chemical., MI, USA). Briefly, 2,4-dinitrophenylhydrazine (DNPH) reacted with protein carbonyl, thus forming a Schiff base to produce the corresponding hydrazone, which could be analyzed spectrophotometrically. Lipid hydroperoxide (which is a better indicator of the degree of lipid peroxidation than other peroxidation products, such as malondialdehyde) was measured by enzyme immunoassay using specific kits (Cayman Chemical., MI, USA).

Single cell electrophoresis (comet assay)

The cells were obtained by washing a small piece of examined tissue with an excess of ice-cold Hank's Balanced Salt Solution (HBSS), then quickly mincing the tissue into approximately 1 mm³ pieces with a pair of stainless steel scissors while immersed in HBSS. After washing several times with cold phosphate-buffered saline (PBS), the minced tissues were dispersed into single cells using a pipette, and resuspended at 1×10^5 cells/ml in ice cold 1X PBS (Ca⁺⁺ and Mg⁺⁺ free). The comet assay was performed as described by Singh [20] using Trevigen's CometAssay™ kit (Catalog # 4250-050-K, AMS Biotechnology GmbH Wiesbaden, Deutschland) as a simple and effective method for evaluating DNA damage in cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates at a slower rate, and remains within the confines of the nucleoid when a current is applied. Briefly, the cells were combined at

Fig. 3. Catalase activity in the hippocampus and cerebellum in different groups. Results shown are mean $\pm$ S.E.M. "a" significant at $p < 0.05$ with control. "b" significant at $p < 0.05$ with ethanol.

Fig. 4. SOD activity in the hippocampus and cerebellum in different groups. Results shown are mean $\pm$ S.E.M. "a" significant at $p < 0.05$ with control. "b" significant at $p < 0.05$ with control.

1 $\times$ 10⁵/ml with molten LM Agarose (at 37°C) at a ratio of 1:10 (v/v) and 75 μ L of the mixture was immediately transferred onto the CometSlide™. Slides were placed flat at 4°C in the dark for 30 minutes and were then immersed in pre-chilled Lysis Solution and left on ice, or at 4°C, for 30 minutes. Slides were immersed in Alkaline Solution for 60 minutes at room temperature, in the dark. Finally, the slides were transferred to a horizontal electrophoresis apparatus. Electrophoresis was carried out at a voltage of 1 Volt/cm (300 mA) for 20 minutes using Alkaline Electrophoresis Solution (pH >13 (300 mM NaOH, 1 mM EDTA)). The slides were treated with 70% ethanol for 5 minutes, and then air dried. They were then stained using 1:10000 diluted SYBR® Green I Staining Solution (10,000x concentrate in DMSO) and examined by epifluorescence microscopy. (Fluorescein filter, excitation and emission are 494 nm and 521 nm, respectively). 100 cells from each slide were blindly analyzed by three independent observers and the results were expressed as a percentage of the comet-positive cells "mean $\pm$ SD". All the cells with different sized tails were considered as comet-

positive cells (fig 6). Hydrogen peroxide treated cells were used as a positive control.

Statistical analysis

One way analysis of variance (ANOVA) was used to compare the activities of SOD, catalase, LPO and protein carbonyl levels among groups. In each test, the data were expressed as the mean $\pm$ SEM and $p < 0.05$ was accepted as statistically significant. The percentage of comet-positive cells were analyzed using the χ^2 test and analysis of variance (ANOVA).

Results

As shown in fig 1, chronic administration of ethanol in pregnant rats resulted in a massive increase in the levels of protein carbonyl in the hippocampus and cerebellum

Fig. 5. Percentage of comet-positive cells in the hippocampus and cerebellum of different groups. “a” significant at $p<0.01$ with control. “b” significant at $p<0.01$ with ethanol.

Fig. 6. Comparison between comet-positive cells and comet-negative cells. (* comet-negative cells, ^ comet-positive cells).

of the offspring compared with controls ($p<0.005$). Treatment with lipoic acid returned the protein carbonyl levels to those of the controls.

The amount of LPO (fig 2) in the hippocampus and cerebellum of the ethanol-treated group was significantly higher than in controls ($p<0.05$). There was no significant difference between the ethanol-lipoic acid treated and control groups ($p>0.5$).

Catalase activity in the hippocampus of the ethanol-treated group was significantly decreased compared to the control group ($p<0.05$), but this activity was restored in the lipoic acid treated group ($p<0.05$). No significant differences were found in the catalase activity of cerebellum between the different experimental groups (fig 3).

As shown in fig 4, the level of SOD was significantly decreased in the cerebellum of both the ethanol and ethanol-lipoic acid treated groups relative to the control group ($p<0.05$). There were no significant differences in the SOD activity of the hippocampus between the three groups ($p>0.5$).

As shown in fig 5, DNA damage, as measured by the percentage of comet-positive cells, was significantly increased in the hippocampal and cerebellar tissues of the rats whose mothers received ethanol, when compared with controls ($p<0.01$). In comparison to the ethanol-treated group, the numbers of comet-positive cells were significantly decreased in the lipoic acid-ethanol treated rats ($p<0.01$), although the number of comet-positive cells was still significantly higher than in the control

group ($p < 0.05$).

Figure 6 shows a comparison of comet-positive cells with comet-negative cells.

Discussion

The results of the present study show that prenatal and postnatal ethanol exposure from GD7 to PND21 at a dose of 4.5 g/kg/day caused significant increases in protein oxidation, lipid peroxidation and DNA damage in the hippocampus and cerebellum of the offspring. Although a limited number of studies have shown alcohol-induced alterations in some measures of oxidative stress in the brain, such as lipid peroxidation and oxidative DNA damage, this is the first *in vivo* study to demonstrate that alcohol exposure during pregnancy increases protein carbonyl formation in the developing hippocampus and cerebellum. In fact, this is one of the few studies to show that alcohol induces oxidative stress in the developing hippocampus and cerebellum, and to our knowledge, the only study that reveals that the occurrence of oxidative stress coincides with DNA damage in these tissues. The brain is more vulnerable to oxidative damage due its high oxygen consumption, and due to the presence of high levels of polyunsaturated fatty acids (PUFAs) [21]. Moreover, this vulnerability could also be associated with the abundance of redox active transition metal ions, and the relative dearth of an antioxidant defense system in the brain [22]. The desire to elucidate the mechanism by which ethanol exposure causes oxidative stress and free radical production (see the introduction) motivated us to investigate these areas. In the current study, we showed that ethanol significantly stimulates LPO levels in the hippocampus and cerebellum of offspring from mothers exposed to alcohol. Since lipid peroxidation is a self-propagating chain reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage and disease, especially in PUFA-rich brain tissue [23]. Gebiki and Gebiki 1993 reported that free radicals induced the formation of the protein peroxides [24]. In addition, when the amount of superoxides produced overwhelms the SOD/catalase system, this can lead to the damage of cell membranes, DNA, and cellular proteins [25]. Our study has supported this notion, and revealed an increase in LPO and protein carbonyl levels, and a marked increase in DNA damage in the hippocampus and cerebellum of the offspring from mothers exposed to alcohol.

Oxidative modification of proteins may lead to the structural alteration and functional inactivation of many

enzymes [26]. In view of the fact that the formation of protein carbonyl groups is orders of magnitude greater than other oxidative modifications, the protein carbonyl levels have become the most widely used marker of protein oxidation during oxidative stress, aging, and disease. This is also probably one of the reasons why we identified an ethanol-induced increase in protein carbonyls in this study.

In the current study, we found that alcohol exposure-related increases in protein carbonyl and LPO could be restored to their normal levels by lipoic acid treatment. Lipoic acid is a naturally occurring dithiol compound that possesses diverse pharmacologic and antioxidant properties [28, 29]. As an antioxidant, lipoic acid chelates transitional metal ions, increases the levels of cytosolic glutathione and vitamin C, and prevents associated toxicity as a result of their loss. Lipoic acid is also an effective scavenger of free radicals in both aqueous and lipid phases [29]. Mouse hippocampal HT22 cells co-incubated with ethanol and lipoic acid exhibited a dramatic decrease in ethanol-mediated neurotoxicity and intracellular protein oxidation [30]. The current study also indicates that ethanol induces massive DNA strand breaks in the developing hippocampus and cerebellum. This is in agreement with a previous report, in which ethanol-induced DNA breaks were observed when rat hepatocytes were incubated in cell culture, and in rat brain cells following alcohol exposure [31-33]. While the cytotoxic effects of ethanol on brain tissue are not entirely understood, two possibilities should be considered [9]. First, ethanol can exert its cytotoxic effects through its lipid soluble properties and therefore displays its biological effects by physically acting as a denaturing or disaggregating agent in cellular macromolecular organization. Second, the cytotoxic effects of ethanol are linked to its metabolic fate, and are most probably are mediated by oxygen-dependent generation of free radicals. There is considerable evidence that free radicals may be formed during cell respiration, and when the rate of electron transport is increased, there is an increase in the formation of these molecules [34]. According to the results of this study, the latter possibility is a plausible explanation for alcohol-induced DNA damage. Our findings show that lipoic acid treatment significantly decreased DNA damage compared with the group treated with ethanol alone, however, the DNA damage in the lipoic acid treatment group is still significantly higher than in controls. Contrary to the hypothesis, our findings suggest that additional mechanisms besides oxidative stress contribute to ethanol-induced DNA damage. Finally, in this study,

the catalase and SOD activities in the hippocampus and cerebellum showed different patterns of change that are oppose to the patterns of lipid peroxidation and protein oxidation. There was no difference in the SOD activity in the hippocampus and the catalase activity in the cerebellum between any of the groups. However, in the cerebellum, SOD activity in the ethanol- and lipoic acid-treated groups showed a significant decrease compared with the control. In the hippocampus, catalase activity was increased compared with the control, and catalase activity was returned to the level of the controls after lipoic acid treatment. The results from previous studies relating the effects of acute ethanol administration upon antioxidant enzyme activities in rat brain are conflicting [35, 36]. These discrepancies may result from variations in experimental species, ethanol doses and protocols. However, the antioxidant defense fails either due to the overproduction of free radicals, or the decreased activities of scavenging enzymes, or a combination of both, resulting in lipid peroxidation.

Although it has been reported that the lipoic acid is an effective protecting antioxidant in the neuronal tissues [37], but it has been also observed that vitamin E

prevents ethanol induced brain damages in developing rats potently [38]. Therefore, it seems that common property of the vitamin E and lipoic acid as natural antioxidants plays a crucial role in their protective effects against ethanol induced pathologic processes in the brain but the different patterns of changes in biochemical markers of oxidative stress, antioxidative defensive system and DNA damages reveal that an additional mechanisms besides oxidative stress are contributed to the observed changes and different molecular targets are being affected in the context of anti oxidative defensive system probably in a “tissue-specific” manner.

In conclusion, our results clearly demonstrate that administration of lipoic acid during gestation and lactation improve ethanol-induced oxidative stress by decreasing protein oxidation and lipid peroxidation. Furthermore, lipoic acid partially alleviated the ethanol-induced DNA damage in developing hippocampus and cerebellum. Further research is required to elucidate the detail of the mechanisms by which alcohol produces these brain deficits, in order to develop feasible prevention or treatment strategies for FAS.

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