Abstract — Acetaldehyde, the first metabolite of ethanol, has been shown to be capable of binding covalently to liver proteins in vivo, which may be responsible for a variety of toxic effects of ethanol. Acetaldehyde–protein adducts have previously been detected in the liver of patients and experimental animals with alcoholic liver disease. Although a role for acetaldehyde as a possible mediator of ethanol-induced neurotoxicity has also been previously suggested, the formation of protein–acetaldehyde adducts in brain has not been examined. This study was designed to examine the occurrence of acetaldehyde–protein adducts in rat brain after lifelong ethanol exposure. A total of 27 male rats from the alcohol-prefering (AA) and alcohol-avoiding (ANA) lines were used. Four ANA rats and five AA rats were fed 10–12% (v/v) ethanol for 21 months. Both young (n = 10) and old (n = 8) rats receiving water were used as controls. Samples from frontal cortex, cerebellum and liver were processed for immunohistochemical detection of acetaldehyde adducts. In four (two ANA, two AA rats) of the nine ethanol-exposed rats, weak or moderate positive reactions for acetaldehyde adducts could be detected both in the frontal cortex and cerebellum, whereas no such immunostaining was found in the remaining five ethanol-treated rats or in the control rats. The positive reaction was localized to the white matter and some large neurons in layers 4 and 5 of the frontal cortex, and to the molecular layer of the cerebellum. Interestingly, the strongest positive reactions were found among the ANA rats, which are known to display high acetaldehyde levels during ethanol oxidation. We suggest that acetaldehyde may be involved in ethanol-induced neurotoxicity in vivo through formation of adducts with brain proteins and macromolecules.

INTRODUCTION

Although the adverse effects of chronic alcohol consumption on the liver as well as on the central nervous system (CNS) are well known, the molecular mechanisms by which long-term alcohol consumption induces tissue toxicity have not been established (Butterworth, 1995; Lieber, 1995; Eckardt et al., 1998). Acetaldehyde, a highly reactive metabolite of ethanol, is able to form covalent acetaldehyde–protein adducts (APA) with several proteins and nucleophиль biomolecules (McKinnon et al., 1987; Lin et al., 1988; Jennett et al., 1989; Nicholls et al., 1992). APA production has recently been implicated in the pathogenesis of ethanol-induced hepatotoxicity. APAs have been detected in livers of ethanol-exposed rats (Lin et al., 1988, 1993; French et al., 1993; Nicholls et al., 1994; Niemelä et al., 1994, 1998), micropropis (Niemelä et al., 1995) and from human alcoholics with liver disease (Niemelä et al., 1991; Holstege et al., 1994). Studies in vitro have indicated that even relatively low concentrations of acetaldehyde can react with liver (Jennett et al., 1989) and brain microtubular proteins and inhibit microtubule polymerization (McKinnon et al., 1987; Smith et al., 1989). Although a role for acetaldehyde in creating harmful effects of ethanol in the central nervous system has previously been suggested (Hunt, 1996), there is as yet no evidence to indicate that APAs could be formed in ethanol-exposed brain tissue in vivo.

The present work was therefore performed to investigate whether APAs are formed in the brain of rats chronically exposed to ethanol. The brain and liver samples, which were used for comparison, were obtained from alcohol-prefering (AA) and -non-prefering (ANA) rats after lifelong (21 months) exposure to alcohol. AA and ANA rat lines are known to differ with respect to their voluntary ethanol consumption and ethanol metabolism, the latter rat line usually displaying higher acetaldehyde levels during ethanol oxidation (Eriksson, 1973).

MATERIALS AND METHODS

Animal model

The present animal model and the protocol for ethanol administration have been previously described in detail (Rintala et al., 1997, 1998). In short, a total of 27 male rats, 15 rats from the AA line (Alko, Alcohol) and 12 rats from the ANA line (Alko, NonAlcohol) were used. AA and ANA lines of rats originated from selective outbreeding for high (AA-line) and low (ANA-line) levels of voluntary ethanol consumption (Hilakivi et al., 1984; Aalto, 1986). The groups were divided into subgroups as follows: AA line, ethanol-fed animals (n = 5); AA line, young controls (n = 5); AA line, old controls (n = 5); ANA line, ethanol-fed animals (n = 4); ANA line, young controls (n = 5); ANA line, old controls (n = 3).

Ethanol feeding

The ethanol-exposed rats were continuously given 12% (v/v) ethanol from 3 months to 24 months of age, except for the 3 weeks self-selection period at the beginning and at the end of the exposure, when the rats were allowed a free choice between 10% ethanol and tap water, in order to measure their voluntary ethanol consumption (Rintala et al., 1997, 1998). A week before the end of the study, the EtOH rats were withdrawn from ethanol. The young (3 months) and old (24 months) control rats had only water to drink. The control rats were subgrouped into old and young to exclude age-related
accumulation of endogenous APAs. Individual ethanol consumption (g of ethanol/kg body weight) was obtained from rats housed in single cages at the beginning and at the end of the experiment. Food [RM1(E)QC:SDS, Witham, UK] was available for all animals at all times. Prior to decapitation, the rats were anaesthetized with sodium pentobarbital (60–120 mg/kg intraperitoneally).

All the experiments described in the present work were carried out in compliance with institutional guidelines and were in full compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals. The research protocol was approved by the Institutional Animal Care and Use Committee of Alko Research Laboratories (National Public Health Institute of Finland).

Antibody production and immunohistochemical methods

Polyclonal antibodies recognizing reduced acetaldehyde-derived epitopes in protein adducts were raised in rabbits by subcutaneous injections of acetaldehyde-bovine serum albumin (BSA) conjugate (1 mg) prepared as described previously (Niemelä et al., 1991). Three booster injections were given with 0.5 mg of the conjugate at 3-week intervals. The animals were bled at 2-week intervals after the second immunization and the anti-acetaldehyde adduct serum was cross-adsorbed on human plasma protein–acetaldehyde conjugate linked to Sepharose 4B (Pharmacia, Uppsala, Sweden).

The right side of the cerebellar hemisphere, frontal cortex and part of the liver were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at 4°C for 24 h and cryoprotected with sucrose (10%, 20%, and 30% sucrose in PBS). For immunohistochemistry the sections were stained by the biotin–streptavidin complex method, employing the following steps: (1) pre-treatment of the cryo-sections with 3% hydrogen peroxide for 5 min followed by rinsing in PBS for 5 min; (2) pre-treatment with undiluted cow colostral whey (Hi-Col, Oulu, Finland) for 40 min to block non-specific binding followed by rinsing in PBS; (3) incubation for 1 h with a 1:200 dilution of the anti-acetaldehyde adduct antiserum in 1% BSA–PBS followed by washing in PBS three times for 10 min; (4) treatment with cow colostral whey for 40 min followed by rinsing in PBS; (5) incubation for 1 h with biotinylated affinity-purified goat immunoglobulins to rabbit immunoglobulins (Dakopatts, Glostrup, Denmark), diluted 1:300 in 1% BSA–PBS followed by washing in PBS three times for 10 min; (6) treatment with cow colostral whey for 5 min; (7) incubation for 30 min with a 1:600 dilution of peroxidase-conjugated streptavidin in PBS and washing in PBS three times for 5 min; (8) incubation for 2 min in diaminobenzidine (DAB) (9 mg DAB in 15 ml PBS plus 10 μl of 30% hydrogen peroxide). All incubations and washings were carried out at room temperature, and the sections were mounted in Permount (Fisher Scientific, Fair Lawn, NJ, USA). The stained sections were evaluated and scored for the intensity of the reactions as follows: 0, no reaction, (+) (= 0.5), scanty reaction, (+) (= 1), weak reaction, ++ (= 2), moderate reaction, +++ (= 3), strong reaction. The sections were photographed with a Leitz Aristoplan microscope (Wetzlar, Germany).

Statistical methods

Results are expressed as mean ± SD. Analysis of variance (ANOVA) was used to examine differences between groups in ethanol consumption and body weights. Testing for differences between indexed values was performed by Mann-Whitney test or by Kruskal–Wallis test, as required. The differences were considered significant at P < 0.05.

RESULTS

There were no significant differences in weight gain and body weights at 24 months between the ethanol-treated rats and the corresponding control animals. The ethanol intake data from 1-week follow-up periods at 3 and at 22 months of age (i.e. representing the mean value of 3- and 24-month data) were obtained from single-housed experimental animals. The average daily ethanol consumption was significantly (P = 0.01) higher among the AA rats, compared with the ANA rats (4.4 ± 0.7 vs 3.1 ± 0.3 g/kg/day).

As a result of ethanol feeding, distinct positive immunohistological reactions for acetaldehyde–protein adducts were found from brain, cerebellum, and liver tissue of some individual animals. The data on the occurrence of APAs in frontal cortex, cerebellum, and liver and the ethanol intakes of the individual experimental animals are summarized in Table 1. Two of the five ethanol-fed AA rats showed positive reactions in frontal cortex and cerebellum. In these AA animals, however, only weak positive reactions were found in the liver in four of the five AA rats. Among the four ethanol-exposed ANA rats, two showed positive staining for APAs in brain and three in the liver.

Figure 1 shows the immunohistochemical reactions in the frontal cortex of the ethanol-treated animals. Positive staining was localized to the white matter and some large neurons and myelinated fibre bundles in layers 4 and 5 of frontal cortex (Fig. 1A and B). A fibrillar staining pattern probably representing axons or dendrites of the white matter was also seen (Fig. 1C).

No such staining was observed in the control animals (Fig. 1D and E). Comparisons of the relative staining intensities between the alcohol-fed AA rats (0.75 ± 0.50), ANA rats (0.94 ± 0.77), control AA rats (0.30 ± 0.35), or control ANA rats (0.22 ± 0.21) did not, however, reach statistical significance.

The cerebellum of the ethanol-exposed animals also showed APAs which were localized into the molecular layer of the cerebellum, frontal cortex and liver of the male AA and ANA after 21 months of ethanol intake.

Table 1. Summary of individual ethanol intake and content of acetaldehyde–protein adducts (APA) in the cerebellum, frontal cortex and liver of the male AA and ANA after 21 months of ethanol intake

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Strain</th>
<th>Ethanol intake (g/kg/day)</th>
<th>APA formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Frontal cortex</td>
</tr>
<tr>
<td>1</td>
<td>AA</td>
<td>5.4</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>AA</td>
<td>4.7</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>AA</td>
<td>4.0</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>AA</td>
<td>4.6</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>AA</td>
<td>3.6</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>ANA</td>
<td>3.3</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>ANA</td>
<td>3.5</td>
<td>+</td>
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<tr>
<td>8</td>
<td>ANA</td>
<td>2.6</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>ANA</td>
<td>3.1</td>
<td>–</td>
</tr>
</tbody>
</table>

AA, alcohol-prefering line; ANA, alcohol-avoiding line.

Arbitrary scale: (+), scanty reaction; +, weak reaction; ++, moderate reaction.
FIG. 1. Immunohistochemical demonstration of acetaldehyde-modified epitopes in frontal cortex. Tissue sections were stained with anti-acetaldehyde adduct antibodies as described in Materials and methods. Positive staining occurred in subcortical structures (A–C), in large neurons and myelinated fibre bundles in the white matter. Controls (D) were devoid of specific staining.

FIG. 2. Immunohistochemical demonstration of acetaldehyde-modified epitopes in cerebellum. Tissue sections were stained with anti-acetaldehyde adduct antibodies as described in Materials and methods. (A) Positive staining occurred in the molecular layer, inside some molecular layer interneurons or astrocytes/microglia, and in the endothelium. Controls (B) were devoid of specific staining.

FIG. 3. Immunohistochemical demonstration of acetaldehyde-modified epitopes in liver. Tissue sections were stained with anti-acetaldehyde adduct antibodies as described in Materials and methods. A faint positive staining occurred in the centrilobular hepatocytes (A), and in sinusoids/capillary endothelium (A). Control (B).

Layer (Fig. 2A). Both endothelial cells and interneurons or glial cells of the molecular layer showed also APA immunoreactivity, whereas the granular layer was devoid of specific staining. The Purkinje cells remained mostly negative. The intensities of the cerebellar reactions were highest in the ethanol-exposed ANA rats (1.13 ± 0.78), which were followed by those in the ethanol-fed AA rats (0.95 ± 0.51), control ANA rats (0.56 ± 0.32), and control AA rats (0.35 ± 0.35).

In the present experiments, the ethanol-treated animals showed no significant histopathological alterations in the liver due to ethanol treatment. The immunohistochemical demonstration of APAs revealed scanty staining, which was restricted to zone 3 hepatocytes (Figure 3A), whereas no such reactions occurred in the control animals (Fig. 3B). The intensities of the immunoreactions were slightly, but not significantly, higher in the ethanol-fed ANA rats (0.78 ± 0.30) than in the AA rats (0.60 ± 0.25) (NS).

DISCUSSION

Although the primary mechanisms for the actions of ethanol on the central nervous system and brain tissue are largely unknown, there are several studies which have postulated a role for acetaldehyde, the first metabolite of ethanol, in creating neurotoxicity (Butterworth, 1995; Hunt, 1996; Hamby-Mason et al., 1997; Zimatin et al., 1997). Previous evidence for the role of acetaldehyde in the extrahepatic effects of ethanol has, however, been inconsistent, which may have been due to the lack of knowledge of the circulating concentrations of acetaldehyde and on the activities of ethanol-metabolizing enzymes in brain in vivo (Hunt, 1996).

The present data provide the first evidence of acetaldehyde-induced protein modifications in the brain of experimental animals as a result of lifelong (21 months) ethanol intake. Although preliminary at this time, current data support a role for acetaldehyde and its covalent binding as a mechanism of toxicity upon brain exposure to acetaldehyde in vivo. Interestingly, the strongest individual APA reactions in various tissues occurred in the ANA rats which are also known to display higher concentrations of acetaldehyde upon ethanol oxidation (Eriksson, 1973). Previous studies in vitro have demonstrated that acetaldehyde is able to form adducts with a wide variety of proteins. The primary structures of such condensation products have not been fully established. However, work by Tuma et al. (1987) has shown that free ε-aminolysine groups are important targets for adduct formation. Acetaldehyde also forms adducts with free ε-amino groups and some aromatic amino acids, and to a lesser extent cysteine. At high concentrations of acetaldehyde, several proteins can form adducts, including albumin and erythrocyte proteins (Israel et al., 1986; Tuma et al., 1987; Wehr et al., 1993; Hernández-Muñoz et al., 1989), tubulin (Smith et al., 1989), lipoproteins (Wehr et al., 1993; Lin et al., 1995), or cytochrome enzymes participating in the metabolism of ethanol (Bereens et al., 1988; French et al., 1993; Lieber, 1995). As a consequence, harmful functional and immunological effects may be expected to occur (Mauch et al., 1986; Tuma and Klassen, 1992; Yokoyama et al., 1995; Viitala et al., 1997). Adduct formation with low concentrations of acetaldehyde has been demonstrated with microtubular proteins, which has been suggested to disturb neurotubulin polymerization (McKinnon et al., 1987; Smith et al., 1989).

Previous studies have indicated that acetaldehyde may be produced by catalase in brain (Gill et al., 1992; Hamby-Mason et al., 1997). Homogenates of immature rat brains are able to generate acetaldehyde via catalase-mediated reaction (Hamby-Mason et al., 1997). Studies by Gill et al. (1992) have further indicated that, through the action of catalase, acetaldehyde is also produced in brain during ethanol intoxication. Zimatin et al. (1998) recently demonstrated the accumulation of acetaldehyde from 50 mM ethanol in several major brain regions, including brain hemispheres and cerebellum. Regional differences were also noted as a function of time, indicating that processes other than catalase also contribute to acetaldehyde formation. Apparently, brain aldehyde dehydrogenase also plays a role in regulating the levels of acetaldehyde (Zimatin, 1991). Acetaldehyde production has also recently been demonstrated in cultured astrocytes (Eysseric et al., 1997; Holownia et al., 1999). Astroglial cells appear to be important targets of ethanol toxicity during central nervous system development (Guerr and Renau-Piqueras, 1997). The effects of ethanol on cerebellum are believed to be important for alcohol’s impairing effect on movement; the large Purkinje neurons in the cerebellar cortex possibly being important mediators of ethanol’s action (Chu, 1983). The present data indicate, however, that Purkinje cells were not preferential targets for acetaldehyde adduct formation, which may indicate that alcohol may act directly on Purkinje neurons, or it may act on other cells that in turn influence Purkinje neuronal activity. As the strongest immunoreaction for APAs was seen in the molecular layer of the cerebellum, it is possible that acetaldehyde could react with microtubular proteins of Purkinje
cell dendrites in vivo. This, in turn, could be responsible for the dendritic regression typically seen in the Purkinje cells of ethanol-exposed animals (Pentney, 1995). Interestingly, our study also shows positive APA reaction in the white matter of frontal cortex, an area which has recently been shown to have a markedly decreased volume in chronic alcoholics (Kril et al., 1997).

Previous studies on APAs in the liver have revealed protein adducts from the centrilobular region of the liver from both human alcohol abusers and experimental animals. Hepatic aldehyde adducts are found as a result of chronic ethanol intake in connection with increased serum aminotransferase levels, accumulation of fat and signs of hepatocellular injury (Niemelä et al., 1991, 1995; French et al., 1993; Holstege et al., 1994). The amount of protein adducts is apparently modulated by the diet, since supplementation of the ethanol-containing diet with high fat or iron markedly stimulates the formation of protein adducts. In the present studies, ethanol alone, even after prolonged administration, was found to generate only minimal histopathological alterations and small amounts of hepatic APAs. Thus, it appears that the present type of prolonged ethanol administration may favour adduct formation in brain structures, instead of liver. Although the present data may also support the role of acetaldehyde in ethanol-induced structural brain lesions, the question whether such processes are responsible for neurodegenerative changes such as brain atrophy in chronic alcoholics remains to be clarified in future studies.

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