CHRONIC ALCOHOL FEEDING INDUCES BIOCHEMICAL, HISTOLOGICAL, AND FUNCTIONAL ALTERATIONS IN RAT RETINA

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Abstract — Aims: Ethanol consumption originates a wide spectrum of disorders, including alteration of visual function. Oxidative stress is included among the mechanisms by which alcohol predisposes nervous tissue to injury. Retina, which is the neurosensorial eye tissue, is particularly sensitive to oxidative stress. Methods: In this study we analyze the effect of long-term alcohol consumption on oxidative stress parameters of the rat retina, and its correlation to retinal function, as well as to the expression of the antiapoptotic protein Bcl-2. We also study the protective effect of ebselen, a synthetic selenoorganic antioxidant. Results: Herein we show that ethanol has a toxic effect on rat retina associated with oxidative stress. Decreases in retina glutathione concentration and increases in malondialdehyde content in whole eye homogenate significantly correlate with ERG b-wave decrease and Bcl-2 overexpression. We also show how ebselen is able to prevent all the alterations observed. Conclusion: Chronic ethanol consumption induces oxidative stress in rat retina associated with an impairment of ERG and Bcl-2 overexpression, suggesting a role for glial cells. All these alterations in the rat allow the proposal of an alcoholic retinopathy in this species.

INTRODUCTION

Alcohol consumption has a high prevalence in most countries and it is the drug with the highest use and abuse in many societies, also in Spain (Grant, 1997; Sanchez, 2002; Alvarez et al., 2006), causing numerous social and medical problems, including serious damage on central nervous system function. Ethanol exerts its deleterious effects metabolically via oxidative and nonoxidative pathways (Bondy and Guo, 1995), involving free radical production and lipid peroxidation (Sun et al., 2001; Ramachandran et al., 2003), potentially leading to an imbalance between oxidants and antioxidants in favor of the former, resulting in an increased oxidative stress (Sies, 1985). Thus, chronic ethanol consumption produces an increase in lipid peroxidation products such as malondialdehyde (MDA) and a decrease in antioxidant factors, such as glutathione (GSH) and its related enzymes (Albano, 2006), which can eventually induce apoptosis-mediated cell death (Yip and Burt, 2006).

In brain, long-term ethanol intake produces a decrease in GSH content and GSH/GSSG (oxidized glutathione) ratio (Calabrese et al., 2002; Agar et al., 2003), as well as an increase in TUNEL positive cells that could be due to an ethanol-related overproduction of reactive oxygen species (ROS) (Ikegami et al., 2003). Similarly, other groups have reported that in liver, alcohol impairs the antioxidant defense system resulting in mitochondrial damage and apoptosis (Seitz and Stickel, 2006).

In the visual system, it is known that long-term ethanol consumption causes clinical manifestations that have been attributed to nutritional deficits rather than a direct effect of ethanol (Dunphy, 1969; Samples and Younge, 1981). Our research group has previously reported that chronic alcohol consumption promotes a decrease in GSH and increase in MDA contents in rat optic nerves (Aviñó et al., 2002), involving oxidative stress in ethanol-induced toxicity and discarding the influence of the nutritional stress on the parameters studied.

Retina is the neurosensorial ocular tissue and it is extremely rich in membranes with polyunsaturated lipids (Kagan et al., 1973), which makes it particularly sensitive to oxygenated free radicals and lipid peroxidation (Bazan, 1989). Increasing evidence suggest that oxidative stress contributes to the pathogenesis of many ocular neurodegenerative disorders, such as diabetic retinopathy (Kowluru, 2003), age-related macular degeneration (Belda et al., 1999) or uveitis (Bosch-Morell et al., 1994, 1999, 2002). However, there are few studies reporting on retinal injury related to oxidative stress induce by ethanol consumption.

Retina function has been widely studied by electroretinogram (ERG), a technique used for decades to uncover the mechanisms of retinal physiology and the underlying lesions of different pathologies (Bui et al., 2004, 2005; Phipps et al., 2006), but these are related to alcohol less intensely (Resende et al., 2001). Therefore, since alcohol has been related to oxidative stress, in the present study we analyze the effect of long-term rat alcohol consumption on oxidative stress in the retina and its correlation to retinal function by ERG.

In addition, we study the expression of the antiapoptotic Bcl-2 protein and its correlation to all the parameters described in the last paragraph, since multiple lines of evidence support the fact that oxidative stress can cause cell death via apoptosis (i.e., in the brain and liver of alcoholic animals), and that Bcl-2 may prevent this process by the up-regulation of an antioxidant pathway (Jang and Surh, 2004). In fact, there are previous studies describing how Bcl-2 may have functional significance in ethanol intoxication, since its expression in the brain is changed.

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after short-term ethanol exposure (Inoue et al., 2002). The protective effect of ebselen (2-phenyl-1,2-benziselenazol-3[H]-one), a synthetic selenoorganic antioxidant (Takasago et al., 1997; Parnham and Sies, 2000) has also been studied. This compound has been studied as a potential agent to counteract some of the deleterious effects of ethanol: it is able to protect the liver (Kono et al., 2001), gastric mucosa (Tabuchi et al., 1995), peripheral nerves (Bosch-Morell et al., 1998), and the brain (Herrera et al., 2003; Johnsen-Soriano et al., 2007).

MATERIALS AND METHODS

Animal manipulation
Male Sprague-Dawley rats weighing 250–400 g were used (Harlan Iberica SL, Barcelona, Spain). All animal manipulations were done according to international regulations of the European Economic Community (order 86/608/CEE) and ARVO (Association for Research in Vision and Ophthalmology). Animals were individually caged and kept in a 12 h/12 h light/darkness environment of less than 110 cd/m² luminance with controlled temperature (20°C) and relative humidity (60%).

After 6 weeks of experiment, animals were sacrificed by cervical dislocation, and eyes were immediately enucleated. One eye was frozen at −80°C until processed for the biochemical analysis of malondialdehyde (MDA) and glutathione (GSH). For this purpose lenses were separated and discarded. The other eye was overnight fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.0 and then embedded in paraffin for the immunohistochemical study.

Ethanol treatment
Twenty-four rats were initially divided into two groups receiving either control or alcohol liquid diets (Lieber et al., 1965), and were paired according to weight. Ethanol and control diets were purchased from Bioserv (Frenchtown, NJ, USA), and prepared in liquid form with either ethanol and water (final ethanol concentration, 6.4% v/v), or water alone. These diets have been developed to supply isocaloric intake in both alcoholic and nonalcoholic conditions, by supplementing the latter with dextrinated maltose accordingly (ethanol-derived calories at 6.4% ethanol concentration provides 350 kcal/l). Ethanol group received the ethanol liquid diet ad libitum during 6 weeks, and control animals received the volume of the corresponding pair on the following day (pair-fed control). Daily consumption was recorded for each rat, and it was 72.7 ± 6.2 ml/day for the ethanol group and 74.9 ± 6.2 ml/day for the control group. Body weight was initially recorded daily, and both groups showed a transient decrease during and after the 4-day adaptation period (during which the final ethanol concentration in the diet was reached) (Aviño et al., 2002); thereafter, body weight was recorded weekly. Ethanol blood level was 195–225 mg/dl during the final 3 weeks of treatment, which was measured randomly every week from two rats. The antioxidant ebselen, purchased from A.G. Scientific (San Diego, CA, USA), was administered with the diet (0.1 mg/ml) to six of the ethanol and six of the pair-fed controls.

Biochemical analysis
Eyes were homogenized in 0.2 M potassium phosphate buffer pH 7.0, and frozen at −20°C until used. Before freezing, an aliquot of each sample was acidified with perchloric acid for GSH measure. It has been demonstrated that 97% of the antioxidants content in rat eye homogenates corresponded to the retina (Doly et al., 1992).

Protein concentration was measured according to the method described by Lowry et al. (Lowry et al., 1951) with a modification described by Peterson (Peterson, 1977).

MDA and GSH were measured in eye homogenates by high-pressure liquid chromatography, following the method of Romero et al. for MDA (Romero et al., 1998), and the procedure described by Reed et al. for GSH (Reed et al., 1980).

Electroretinogram
After 6 weeks of ethanol or control treatments, rats were dark-adapted overnight in order to perform ERG as described previously (Miranda et al., 2004, 2006). Under dim red light, rats were anesthetized with ketamine (100 mg/kg body weight) and azeprromazine (2.5 mg/kg body weight). Anesthetic and miadriatic colirium were administrated. An active wire loop electrode was used to record responses, and needle electrodes were placed in the neck and tail of the rat, which served as the reference and ground, respectively. Flash ERG were elicited with 4-ms flashes of white light (mean 4; range 100, intensity 1 = 14 cd/m²), and responses of four flashes were averaged and recorded over 100 ms by using a MacLab computer equipment (Castle Hill, Australia). A blue filter was used. The passing band of the preamplifier was set between 3–50 Hz, with a 100 μV range.

Bcl-2 immunohistochemical analysis
Five-micrometer tissue sections were obtained from paraffin-embedded blocks on microtome, mounted from warm water onto adhesive microscope slides, and dried overnight at room temperature. To start the immunoassay, samples were deparaffinized and rehydrated using standard methods.

For the antigen retrieval, sections were pretreated with citrate buffer (10 mM, pH 6.0) in a preheated water bath at 95–100°C for 20 min; then the slides were kept in citrate buffer for 20 min allowing them to cool to room temperature. After rinsing with PBS 0.02 M, endogenous peroxidase was blocked by incubating slides with 30% H2O2 and methanol for 20 min. Then, sections were rinsed with PBS and incubated with blocking normal goat serum (NGS) for 20 min at room temperature. Thereafter, sections were incubated overnight at 4°C with rabbit anti-Bcl-2 primary antibody (dilution 1:500; Dako, Barcelona, Spain) in PBS containing 0.08% Triton X-100 and 5% NGS. Sections were rinsed with PBS and incubated with 0.4% biotinylated goat anti-rabbit IgG (Dako); after 1-h incubation at room temperature with shaking, sections were rinsed and incubated with the avidin–biotin complex (ABC, Dako) for 1 h. Color was developed by using DAB as substrate (Dako) for 5 min at room temperature. Finally, sections were counterstained with hematoxylin and covered with DPX mounting medium for light microscope analysis. Picture color analyzer software (Saegusa Precision Software, Fukui, Japan) was used to quantify the number of Bcl-2 positive cells, based on their staining, as follows: the intensity of each of the three basic
colors was established for a given area of the sections, and a mean value was calculated (for details cf. Otaka et al., 2002).

**Statistical analysis**

Data are expressed as mean ± standard error of five rats in each group. Statistical significances were assessed by 1-way ANOVA followed by the Student’s t-test. The level of significance was set at $P < 0.05$.

Pearson’s correlation coefficient (R) was used to determine the degree of linear correlation between the parameters studied (MDA, GSH, b-wave amplitude, and Bcl-2 expression).

**RESULTS**

**Biochemical data**

The effect of ethanol treatment on biochemical oxidative stress markers was analyzed in eye homogenates without lens (Figs. 1A and B). After 6 weeks of ethanol exposure, a significant variation on the concentration of the metabolites studied was observed, when compared with their respective pair-fed control animals. The levels of the intracellular antioxidant glutathione (GSH) were significantly reduced (Fig. 1A), whereas the concentration of the lipid peroxidation product malondialdehyde (MDA) showed a significant increase (Fig. 1B). Ebselen administration was able to prevent these alterations (Figs. 1A and B).

**Electroretinogram results**

After 6 weeks of ethanol treatment, b-wave amplitude of the ERG was significantly reduced in ethanol-treated animals compared to controls. The animals treated with ethanol and ebselen did not show this reduction (Fig. 1C).

**Bcl-2 expression**

Six weeks of alcohol consumption caused a significant overexpression of the antiapoptotic protein Bcl-2 in rat retinas when compared to control animals, and this phenomenon was again prevented by ebselen treatment (Fig. 1D). This overexpression occurred apparently in Müller cells (Fig. 2).

**Oxidative stress/b-wave/Bcl-2 correlations**

MDA concentration of each eye were plotted against the ERG b-wave amplitude values (Fig. 3A) and against the Bcl-2 expression levels (Fig. 3B). Both revealed a high correlation coefficient; the former showed a negative correlation and the latter a positive one. Interestingly, when the Bcl-2 expression levels were plotted against ERG b-wave amplitude values and against GSH concentrations of each eye, both showed a negative correlation, again with high correlation coefficients (Figs. 4A and B, respectively).

**DISCUSSION**

Ethanol consumption originates a wide spectrum of disorders, including the alteration of visual function. However, a real alcohol-related optic neuropathy has not been accepted for years, denying an intrinsic effect of ethanol. The vision loss found in many alcoholics has been generally included in a medical profile known as tobacco–alcohol amblyopia (Victor and Dreyfus, 1965; Dunphy, 1969; Samples and Younge, 1981), where a synergistic effect was attributed to alcohol and tobacco consumption associated with a lack of vitamins secondary to a deficient nutrition, which is usual in these patients (Hoyt, 1979). Moreover, although this syndrome has been classified as optic neuropathy, the primary lesion has not actually been localized within the optic nerve, and may possibly be originated in the retina, chiasm or even the optic tracts, and in fact an ethanol-induced macular lesion has also been suggested (Williams, 1984; Behbehani et al., 2005).

**Ethanol-induced retinal oxidative stress**

Alcohol predisposes nervous tissue to injury via multiple mechanisms, including the development of oxidative stress (Sun et al., 2001). Several studies reported that the ROS formed from these systems are important in causing oxidative stress in the central nervous system (Hansson et al., 1990; Warner et al., 1998).
Fig. 2. Immunohistochemical staining of Bcl-2 in retina sections of control (A), ebselen-treated control rats (B), ethanol-fed (C), and ethanol + ebselen–treated rats (D). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Fig. 3. Linear correlation between MDA concentration in eye homogenates without lens and ERG b-wave amplitude (A), and retinal Bcl-2 expression (B).

Fig. 4. Linear correlation between retinal Bcl-2 expression and ERG b-wave amplitude (A) and GSH concentration in eye homogenates without lens (B).

and Gustafsson, 1994), which renders a significant decrease of antioxidant enzymes and an altered GSH homeostasis in brain (Rouach et al., 1997; Calabrese et al., 2002).

The results herein from eyes of ethanol-treated rats show an increase in lipid peroxidation-derived products (MDA), as well as a decrease in the level of endogenous antioxidants (GSH) (Fig. 1A and B). These findings agree with previous studies that reported an alteration of oxidative stress metabolites after long-term administration of ethanol in liver (Oh et al., 1998; Bailey et al., 2001) and brain (Renis et al., 1996; Omodeo-Sale et al., 1997; Calabrese et al., 2002; Johnsen-Soriano et al., 2007), and also with our previous publications that reported an alcohol-induced GSH decrease and MDA increase in peripheral nerve (Bosch-Morell et al., 1998), optic nerve (Avihó et al., 2002), and hippocampus (Johnsen-Soriano et al., 2007), suggesting a direct toxic effect of ethanol related to oxidative stress.

Although brain or liver have been broadly studied as targets of ethanol toxicity, our results also suggest that retina might be another vulnerable area for redox changes induced by alcohol intoxication, with lower threshold levels of ethanol tolerance.
In fact, rod outer segment membranes contain a high amount of long-chain polyunsaturated fatty acids (Anderson and Andrews, 1982), which make them particularly susceptible to oxidative stress.

Are Müller cells challenged by chronic ethanol feeding?

Interestingly, ERG b-wave amplitude values in ethanol group showed a high correlation with MDA levels in eye homogenates (Fig. 3A), which directly relates ethanol-induced oxidative stress to retinal function integrity. These results agree with (Pawlosky et al., 2001), who reported that ethanol originated an increase in both ERG a- and b-wave implicit times and a decrease in b-wave amplitude. Pawlosky’s study was carried out in monkeys that consumed ethanol for a long period of time (5 years), and their brain and retina biopsies revealed a decrease in fatty acids, mainly docosahexaenoic acid (DHA), which is abundant in rod membranes (Aveldano and Bazan, 1972). DHA is required for brain and retina development (deD’Angelo et al., 1977) and has been implicated in excitable membrane function (Litman et al., 2001), memory (Moriguchi and Salem, 2003), photoreceptor biogenesis and function (Wheeler et al., 1975; Scott and Bazan, 1989; Stinson et al., 1991), and neuroprotection (Bazan, 2005). Indeed, DHA has been shown to protect photoreceptors from oxidative stress–induced apoptosis by, among other mechanisms, increasing the expression of the antiapoptotic protein Bcl-2 (German et al., 2006).

It is known that Bcl-2 is highly expressed in glial cells, and previous studies showed that in the adult human retina Bcl-2 is expressed predominantly in Müller glial cells and in the ganglion cell layer (Abu-El-Asrar et al., 2004). Herein we show an increase in the expression of this protein in the retina of alcohol-fed rats in these areas, which, in addition, negatively correlates with the b-wave amplitude of the ERG (Fig. 4A). Since ERG b-wave is known to reflect Müller cell activity (Miller and Dowling, 1970), these data clearly suggest that chronic alcohol administration affects glial cells of rat retina.

Furthermore, the protooncogene Bcl-2, which can protect neurons from ethanol toxicity (Heaton et al., 1999; Moore et al., 1999), may prevent apoptosis via regulation of an antioxidant pathway and is considered to act itself as a free radical scavenger (Jang and Surh, 2004). This could explain our finding showing that the increase of MDA as well as the decrease of GSH concentration in the retina correlates significantly with an increase of Bcl-2 expression in the retina of alcohol-fed rats (Figs. 3B and 4B, respectively). In fact, a decrease of GSH concentration is one of the stimuli that can induce the apoptotic process (Rahman and MacNee, 2000), therefore the overexpression of Bcl-2 could be a defense mechanism, conferring resistance to oxidant injury and preventing apoptosis. Moreover, we have not found differences in the number of cells with picnotic nuclei, positive for Nissl staining, between the retinas of control and alcohol-treated rats (data not shown), suggesting that Bcl-2 could play an important role in maintaining their survival.

Protective effect of the antioxidant ebselen

In this study, we also successfully assayed a treatment with the antioxidant ebselen, which resulted in a prevention of all ethanol-induced alterations observed in this study. These results agree with our previous findings, which demonstrated that ebselen prevented ethanol-induced biochemical and functional impairment in rat hippocampus (Johnsen-Soriano et al., 2007) as well as the impairment of neurogenesis observed in the hippocampus of these animals (Herrera et al., 2003). In conclusion, the present study shows a direct oxidative toxic effect of ethanol on rat retina, which highly correlates with ERG b-wave decrease and Bcl-2 antiapoptotic protein overexpression. It is also shown as to how ebselen is able to prevent all the alterations observed. Thus, these results confirm the role of oxidative stress in the pathophysiology of chronic alcohol-induced retinal damage, which could be understood as an alcoholic retinopathy in this species.

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