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Chronic Ethanol Ingestion-Induced Changes in Open-Field Behavior and Oxidative Stress in the Rat

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HARKANY, T., M. SASVARI AND C. NYAKAS. *Chronic ethanol ingestion-induced changes in open-field behavior and oxidative stress in the rat.* PHARMACOL BIOCHEM BEHAV **58**(1) 195–201, 1997.—The effects of chronic ethanol intoxication on the open-field behavior, on antioxidant enzyme activities, and the degree of lipid peroxidation were investigated. Rats consuming a liquid diet containing 7% ethanol for 4, 7, 14, or 21 days exhibited a significantly decreased ambulation activity, accompanied by a reduced frequency and duration of explorative rearing in an open-field task 4, 7, and 14 days after chronic ethanol ingestion, whereas presumed adaptation to the neurologic effects of ethanol was observed on day 21. Changes in the activities of glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R), and catalase, and in the content of reduced glutathione (GSH) in blood samples were determined by means of biochemical methods. The degree of lipid peroxidation was measured via thiobarbituric acid assays. Chronic ethanol ingestion elicited a significant increase in GSH-Px activity (by a maximum of ~32% on day 14), whereas opposite alterations in GSH-R and catalase activities were recorded (49% of the control value on day 4 and 17% on day 21, respectively). Highly elevated contents of thiobarbituric acid reactive substances reflected extensive lipid peroxidation processes throughout the experiment. These changes indicate that ethanol toxicity induces profound changes in explorative behavior, mediated, at least partly, by changes in the free radical metabolism. © 1997 Elsevier Science Inc.

Blood Catalase Chronic ethanol intoxication Oxygen free radicals Glutathione Glutathione peroxidase Glutathione reductase Lipid peroxidation Open-field behavior Oxidative stress Rat

BASIC molecular pathways of cellular injury, elicited, for example, by acute or chronic ethanol intoxication (21,23,39), ischemia (45), or various neurotoxins, such as β-amyloid peptides (14,19), leading to cell death, involve the generation of free radicals both in the central nervous system and at the periphery. Free radical-induced tissue damage is most commonly mediated via highly reactive oxygen intermediates formed during the reduction of oxygen, including superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$). Indeed, $OH^{\bullet}$ displays a great ability to react with many (if not all) biological molecules, including lipoperoxides, causing alterations in the membrane structure (35). Thus, reactive oxygen species (ROS) contribute to cellular function disturbances, leading to cell death by derangement of biological membranes. Lipid peroxidation may therefore be of major significance in cell injuries produced by free radicals (12,13).

Various antioxidant substances that are unequally expressed in different types of tissues (e.g., neurons, hepatocytes, and erythrocytes) (6,22,23,39) protect against free radical-induced damage. These defense mechanisms range from low-molecular-weight compounds such as glutathione, tocopherols or vitamin E, to complex enzymatic systems. In this respect, superoxide dismutase (SOD; EC 1.15.1.1), the glutathione peroxidase (GSH-Px; EC 1.11.1.9)/glutathione reductase (GSH-R; EC 1.6.4.2) balance, and catalase (EC 1.11.1.6) play major roles in the ROS ($O_2^{\bullet -}/H_2O_2$) metabolism. It is noteworthy, however, that the contributions of GSH-Px and catalase to the processes of H_2O_2 detoxification are claimed to be highly dependent on the H_2O_2 concentration (5).

Evidence has accumulated regarding the role of oxygen-derived free radicals in the pathogenesis of alcohol-induced cellular injury (1), although the molecular mechanisms of

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Ethanol and alcohol are used interchangeably in this article.

ROS generation elicited by ethanol still remain to be ascertained. It likewise remains to be clarified whether $O_2^{\bullet -}$, H_2O_2, or $OH^{\bullet}$ mediates ethanol-induced toxicity, although some findings indicate a critical role for H_2O_2 metabolism (11,43). It is interesting that the effects or the metabolism of acetaldehyde have also been implicated as a source of free radicals (24,40) in the pathogenesis of ethanol toxicity. It has been proposed, therefore, that the ethanol and/or acetaldehyde metabolism (24) can stimulate lipid peroxidation through the formation of free radicals or by exhausting antioxidant substances, leading to oxidative stress both in the liver (7,9) and in extrahepatic tissues, such as the heart or brain (23,25,31).

We have recently demonstrated that repeated ethanol treatment (15) differentially alters the levels of Ca^{2+}-binding proteins (CaBPs) in mouse brain. Furthermore, with the same model of chronic ethanol administration as described in the present account, persistently altered expressions of not only neuronal, but also glial CaBPs, parvalbumin, calbindin-D28k, and S-100 have been demonstrated in a time-dependent manner (Harkany et al., unpublished data). These results lend further support to the specific neurotoxic processes induced by chronic ethanol administration that are mediated (directly or indirectly) by elevation of the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Increased $[Ca^{2+}]_i$, in turn, stresses the generation of ROS (20) and might influence the expression of antioxidant enzymes by stimulating distinct intracellular signaling pathways, which activate different sets of transcription factors (8). Additionally, it is a firmly established notion that mechanisms (e.g. hypoxia or ethanol treatment) causing an increased Ca^{2+} influx impair learning mechanisms (18,21,26,27) and result in abnormal open-field behavior in rats (27), which can be diminished by Ca^{2+}-channel blockade (18,26,27).

A multitude of in vivo and in vitro findings have demonstrated that both acute (33,39,45) and chronic (22,23) ethanol administration lead to a rapid elevation of ROS and to a greater degree of lipid peroxidation in the liver (33,43,45) and brain (23). Furthermore, recent studies have revealed long-term effects of ethanol ingestion on spontaneous and learning behavior in rats (18,21). In the present study, we set out to investigate the possible interaction between altered animal behavior and blood antioxidant enzyme activities in a time-course experiment. The characteristic signs of altered novelty-induced animal behavior were, therefore, assessed in an open-field paradigm after 4, 7, 14, and 21 days of chronic ethanol ingestion. To study the role of free radicals in ethanol toxicity, we determined the alterations in blood antioxidant enzyme activities, particularly GSH-Px, GSH-R, and catalase, and lipid peroxidation processes by means of biochemical methods. Because SOD activity was demonstrated to change only slightly after several months of intoxication using moderate doses of ethanol (7,11), determination of this enzyme activity was not a subject of the present study. Moreover, reduced glutathione (GSH) contents were determined to give further indication on the actual activity of the GSH-Px/GSH-R cycle after chronic ethanol treatment.

METHOD

Animals and Treatment

Thirty-two young male Wistar rats (340–390 g), divided into two groups, were housed individually at least three days prior to the experiment and kept under normal laboratory conditions in a temperature- and light-controlled room (21 ± 2°C, 12/12 h light/dark cycle, lights on at 0700). The rats were then fed ethanol in a liquid diet, or a control liquid diet, for 4, 7, 14, or 21 days, as described previously in detail in mice (32) with minor modifications, the regimen being adapted to rats. Briefly, the group of ethanol-ingested rats was given a 7% ethanol-containing diet supplemented by vitamins (3 g/l), whereas control animals were offered the same diet, except that ethanol was substituted by an amount of sucrose equivalent to the calories available in ethanol. This regimen of ethanol administration has been shown to produce a functional alcohol tolerance and physical dependence in ethanol-treated mice (32) and rats by ourselves (data not shown) and others (36). A significant difference in weight gain was not observed between the ethanol-fed (373.72 ± 14.9 g) and control (421.76 ± 37.5 g) groups at the end of the treatment. The daily average ethanol intake of ethanol-treated rats was 38.81 ± 5.6 ml of the 7% ethanol-containing diet, which is equivalent to 5.88 ± 0.8 g of absolute ethanol/kg body weight (Fig. 1). Access to ethanol in the drinking fluid was maintained up to the termination of the experiment in order to prevent any possible withdrawal stress. Using a similar procedure of ethanol administration, we have previously demonstrated (15) that moderate, repeated ethanol intoxication elicits characteristic changes in the immunocytochemically detectable expression of CaBPs in the mouse brain.

After the treatment period, the rats were deeply anesthetized with chloral hydrate (375 mg/kg body weight; Sigma, St. Louis, MO, USA) and blood samples were collected directly from the heart by cardiac puncture. To prevent coagulation, 1 mg/ml disodium ethylenediaminetetraacetate (EDTA, Sigma) was added immediately to all blood samples, which were then stored at 4°C until further processing. Baseline determinations were performed on the initial day of the experiment, using individually caged animals. All experiments on rats were performed in strict compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (1985).

Open-Field Activity

The novelty-induced activities of ethanol-fed and control rats were measured as described initially by Gray and Lalljee

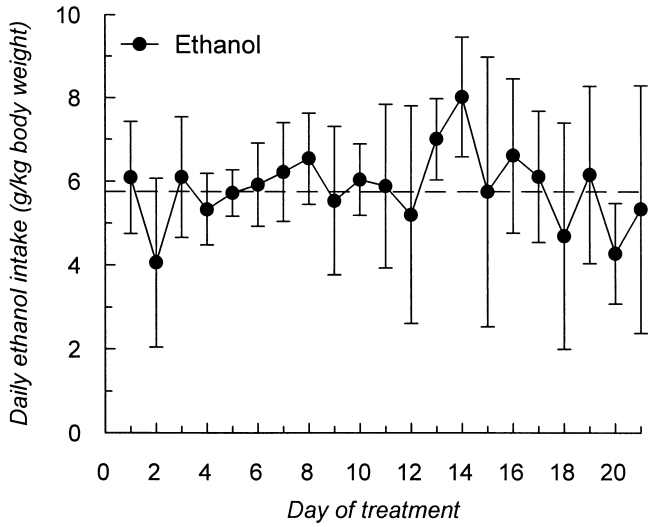

FIG. 1. Changes of daily ethanol intake by ethanol-fed rats during 21-day chronic ethanol ingestion. The average of daily ethanol consumption (dashed line) was 38.81 ± 5.6 ml of a 7% ethanol containing diet, which is equivalent to 5.88 ± 0.8 g of absolute ethanol/kg body weight. Data are expressed as means ± SD.

(10) and modified slightly by Nyakas et al. (26,27) in an open-field test box consisting of a circular arena 80 cm in diameter, which was divided into 20 sectors by faint brown concentric and radial lines, and surrounded by a 45-cm high aluminum wall. Illumination was provided by a 30 W bulb, positioned 60 cm above the floor of the apparatus. All measurements were obtained in a darkened and quiet room in the morning (10:00). The test was started by placing the rat in the center of the arena. Each test lasted for 3 min, during which (1) the latency to start exploration, (2) the degree of horizontal ambulation (the number of line-crossings), (3) the number of times and (4) the duration during which the rat simultaneously raised both forepaws off the floor of the apparatus (rearing), and (5) the number of fecal boli deposited (defecations) were recorded.

Biochemical Determinations

Blood samples from ethanol-fed and control animals were processed simultaneously. Erythrocytes and plasma samples were separated by immediate centrifugation at 3,000 × *g* for 10 min at 4°C and were used further separately. After 3 × 10 min washing with ice-cold physiological saline, erythrocytes were hemolysed with distilled water to a 10 × dilution and aliquots were stored at −20°C until further processing. The hemoglobin (Hb) content of all hemolysates was determined by using the MINOS SPE automatic system (Roche, France). GSH-Px activity was measured spectrophotometrically (412 nm; Specol 220, Zeiss, Germany) in 10 × diluted hemolysate by applying 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) in the presence of cumene hydroperoxide as cosubstrate (37). The activity of GSH-Px was expressed in μmol GSH oxidized/min/g Hb at 37°C. GSH-R activity was determined in 10% hemolysates as described previously (4). Briefly, the oxidized form of glutathione is reduced to GSH by GSH-R in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which serves as the H^+ donor in the chemical reaction. The oxidation of NADPH (the characteristic shift in the UV spectrum) was followed spectrophotometrically (340 nm, Specord UV-VIS) throughout a 3-min period, indicating the GSH-R activity. The activity of GSH-R was expressed in μmol NADPH oxidized/min/g Hb at 37°C. The GSH content of the same hemolysate was similarly measured spectrophotometrically (412 nm, Specol 220), with DTNB (41). The amount of GSH in the hemolysate was expressed in nmol/g Hb. A method (3) involving back-measurement of the rate of H_2O_2 consumption at 240 nm (Specord UV-VIS) and 25°C was employed for quantitative determination of catalase activities in 100 × diluted hemolysates after chronic ethanol ingestion. The activity of catalase was expressed in g H_2O_2 hydrolyzed/min/ml hemolysate at 25°C. The degree of lipid peroxidation was established by estimation of the thiobarbituric acid reactive substance (TBARS) contents of both erythrocytes (28,29) and plasma (44). In brief, the TBARS contents were determined as described by Ohkawa et al. (28) and Yagi (44) with the use of thiobarbituric acid (Merck, Darmstadt, Germany) by means of spectrophotometry at 532 nm (Specol 220). The erythrocyte and plasma TBARS contents were expressed in nmol/g Hb and nmol/ml plasma, respectively.

Statistical Analysis

Changes in body weight were analyzed using paired Student *t*-test and expressed as mean ± SD. The results of open-field experiments were evaluated statistically with the non-parametric Kruskal–Wallis test (MINITAB®, Release 9.2, 1993, Minitab Inc., State College, PA, USA) and are presented as medians. Data on biochemical parameters, including enzyme activities and GSH and TBARS contents, are presented as percentages of the control level ± SD. The two-tailed Student *t*-test was applied for statistical evaluation. A p level of < 0.05 was taken as indicative of statistical significance for the tests; this was calculated with a computer program for an IBM PC (Excel 5.0, 1993, Microsoft Co., Redmond, WA, USA). The rates of changes in enzyme activities were calculated by linear regression analysis, followed by analysis of variance (MINITAB®, Release 9.2, 1993).

RESULTS

Behavioral Analysis

The behavioral activities of ethanol-treated and control rats in response to a novel environment were compared in the open-field after 4, 7, 14, or 21 days of chronic ethanol ingestion. Analysis of our data obtained by measurement of the latency to the start of locomotion revealed a marked impairment of explorative behavior after chronic ethanol ingestion, manifested in significantly increased start latencies (7 s [ethanol] vs. 2 s [control] after 21 days of chronic ethanol ingestion; Fig. 3A). Chronic ethanol feeding resulted in a dramatically decreased horizontal locomotor activity (ambulation), by a maximum of 62% of the control value after 4 days of treatment ($p < 0.01$; Fig. 2A); this was maintained, albeit less significantly ($p < 0.05$), up to 14 days of treatment, whereas the difference in line crossings between the ethanol-fed and control groups disappeared after 21 days of chronic ethanol intoxication (55.0 [ethanol] vs. 55.5 [control] ambulation score; Fig. 2A). In parallel with the changes in horizontal locomotor movements, similar effects of chronic ethanol treatment were recorded on the frequency and duration of rearing activity (Figs. 2B and 3B). The suppressant effect of chronic ethanol ingestion on exploratory behavior was most apparent after 7 and 14 days of treatment ($p < 0.01$, $p < 0.05$; Figs. 2B and 3B). Interestingly, however, an overshoot in the duration of rearing activity (22s [ethanol] vs. 13s [control]) was observed on day 21 of ethanol treatment. The number of fecal boli deposited in the open field revealed a persistent, significantly diminished stress reaction of ethanol-fed animals in the illuminated arena as compared with controls ($p < 0.01$; Fig. 2C).

Biochemical Studies

To determine whether chronic ethanol ingestion induces oxidative stress in the rat, changes in antioxidant enzyme activities and the extent of lipid peroxidation were measured biochemically in blood samples. Chronic ethanol administration resulted in opposite alterations in the blood GSH-Px and GSH-R activities (Fig. 4A). Increased GSH-Px activities were recorded throughout the experiment, reaching the level of significance only after 14 days of treatment (by 32.8% of the control value, $p < 0.05$; Fig. 4A), whereas chronic ethanol intoxication elicited a dramatic decrease in GSH-R activity ($p < 0.01$; Fig. 4A), with the highest reduction (49.8% of the control value) after 4 days of intoxication. The blood GSH content exhibited a slight elevation (Fig. 4B), while blood catalase activity persistently decreased ($p < 0.05$; Fig. 5A) throughout the experiment. The extent of formation of TBARS, involving mainly malonyl dialdehyde, was determined in both erythrocytes and plasma. As shown in Fig. 5B, highly increased TBARS levels were found in both fractions, indicating an in-

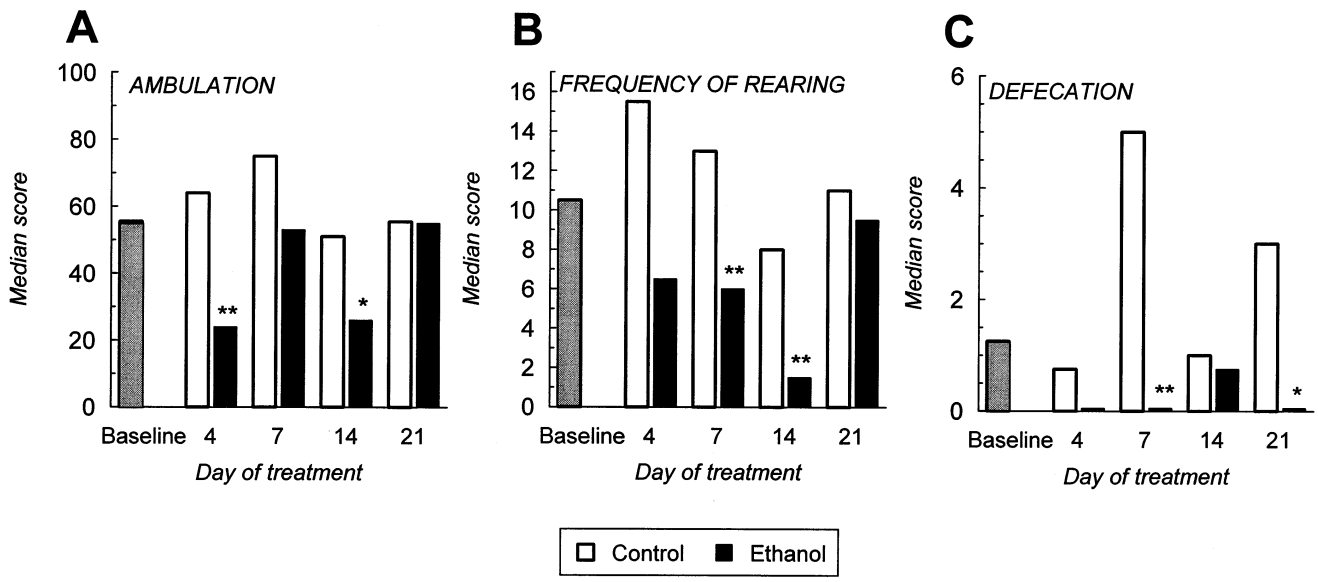

FIG. 2. Intensity of horizontal locomotion (ambulation; A), rearing (B), and defecation (C) of control and ethanol-fed animals in the open-field after 4, 7, 14, or 21 days of chronic ethanol intoxication. Four control and four ethanol-treated rats were used in each time point to determine behavioral activities in the open-field. *$p < 0.05$; **$p < 0.01$ (Kruskal–Wallis test) for ambulation, frequency of rearing, and defecation, respectively. Data are reported as medians.

creased degree of lipid peroxidation relative to the controls ($p < 0.05$).

DISCUSSION

Changes in Animal Behavior

Chronic ethanol ingestion exerts profound effects on the novelty-induced explorative behavior of ethanol-fed rats. The delay in starting exploration in the open-field test was significantly higher, whereas the horizontal motor activity and the frequency and duration of rearing were significantly decreased in animals subjected to chronic ethanol intoxication relative to the controls. The number of fecal boli deposited in the open-field demonstrated a significantly attenuated stress reaction of ethanol-fed rats under the illuminated conditions of the arena, as compared with the controls. It is worth noting that the differences in ambulation and rearing activities were reduced after a

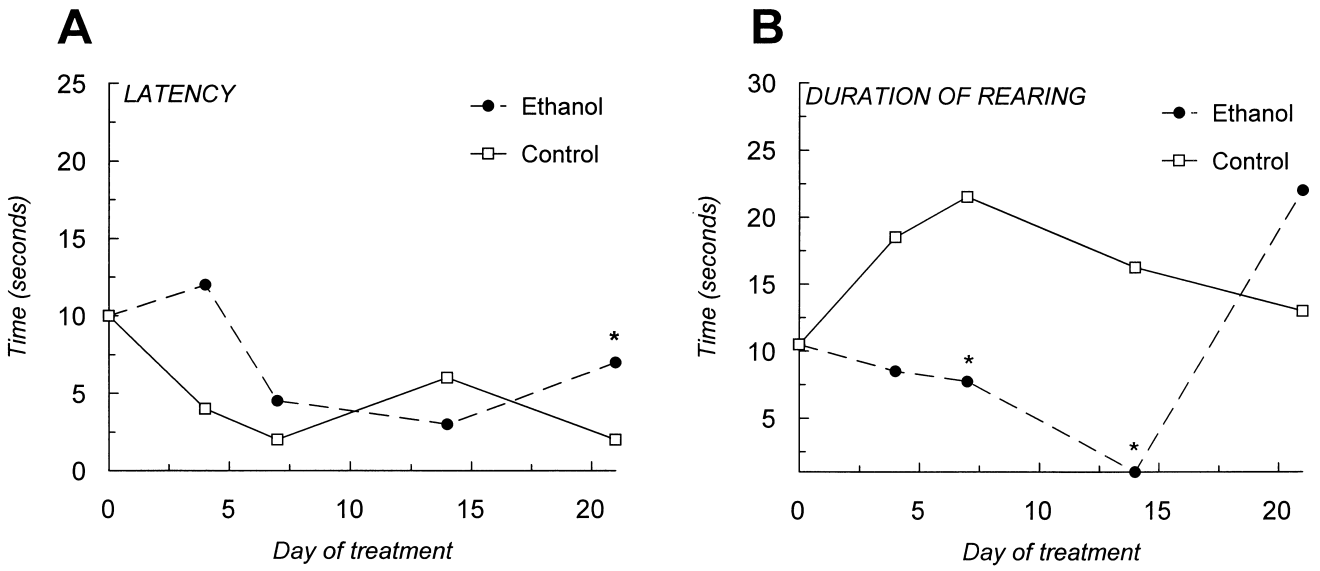

FIG. 3. Changes in the latency to start exploration (A) and duration of explorative rearing (B) in a cylindrical open-field task as the result of 4, 7, 14, or 21 days of chronic ethanol intoxication. Each value is the median of the results on four animals. *$p < 0.05$ (Kruskal–Wallis test) vs. the control group.

21-day treatment period, which may reflect adaptation to and compensation of the direct effects of ethanol. The present results indicate that the severity of these behavioral changes correlates with the degree of ethanol toxicity as they were recorded in the open-field test, although some of these alterations decreased after 21 days of chronic ethanol treatment.

Recent findings on the permanently altered expression of CaBPs (15) and on the beneficial effects of the administration of the Ca^{2+}-channel antagonist nimodipine (18) support the hypothesis that an extensively altered $[Ca^{2+}]_i$ may play a pivotal role in ethanol-induced neurotoxicity and behavioral dysfunctions. Moreover, the involvement of ROS in the decline of learning and memory functions in neurodegenerative disorders and in aging (2) are well established. On the basis of the present results and previous studies involving the use of immunocytochemistry (15) or biochemical methods [Sasvari et al., unpublished observations; (23)], we postulate simultaneous alterations in the intracellular Ca^{2+} homeostasis (15,18) and the generation of ROS-mediated ethanol toxicity. The results of Mattson et al. (20) are likewise corroborated by the present findings, because direct interactions of elevating $[Ca^{2+}]_i$ and a rapidly increasing generation of ROS (and vice versa) were established in vitro.

Oxidative Stress in Erythrocytes

It has long been known that the basic molecular mechanisms of cellular injury leading to cell death involve the generation of ROS (20,23,39). The protection of the normal cellular function is primarily based on the presence, increased expressions, and, therefore, elevated activities of antioxidant defense systems, such as the glutathione cycle, catalase, Zn/Cu-, or Mn-SOD (12,13,42,43). In view of the results of many previous in vitro (17,30) and in vivo studies (15,16,23), both acute and chronic ethanol administration have been firmly demonstrated to exert toxic effects on several types of tissues, although the particular pathways of action of ethanol are still under investigation. The characteristic degree of tissue ethanol metabolism has been claimed to be a key factor, and in this respect hepatic (34,38,43) and brain (16,23,34) dysfunctions have been studied extensively. Because the intrinsic antioxidant defense systems of the blood, located mainly if not exclusively in the erythrocytes, act as the primary barrier against tissue injury mediated directly by ethanol or its metabolites (e.g., acetaldehyde) after their absorbtion in the gut, in the present series of experiments the rates of alterations in the activities of ROS-scavenging systems in the blood were examined after chronic ethanol ingestion. Additionally, the level of lipid peroxidation was determined in order to assess whether ethanol accelerates the destruction of the membranes of the erythrocytes. Moreover, because of its considerable volume and immediate contact with ethanol metabolites, the blood may make an evident contribution to the prevention of ethanol toxicity.

In response to chronic ethanol feeding, the GSH-Px activity increased, whereas the GSH-R activity was reduced significantly, although the activity profiles of these enzymatic changes differed considerably. The change in GSH content exhibited a similar pattern to that in GSH-Px activity. The results of previous studies (42) lead us to presume that changes in the amount of GSH in hemolysates may indicate an increased level of intracellular de novo synthesis of GSH by the rapidly inducible GSH-producing enzyme GSH-synthetase (EC 6.3.2.3). Detailed analysis of these changes showed that the GSH content reached its maximum after 14 days of chronic ethanol intoxication. The slight, early depletion of the GSH content observed after 4 days of treatment might reflect temporary perturbations of GSH precursor supplementation. The changes in GSH-Px activity were found to correlate closely with those in its substrate (GSH), although the level of significance was reached only after 14 days of treatment. GSH-R reduces the oxidized form of glutathione to the active, ROS-scavenging GSH. Chronic ethanol ingestion signifi-

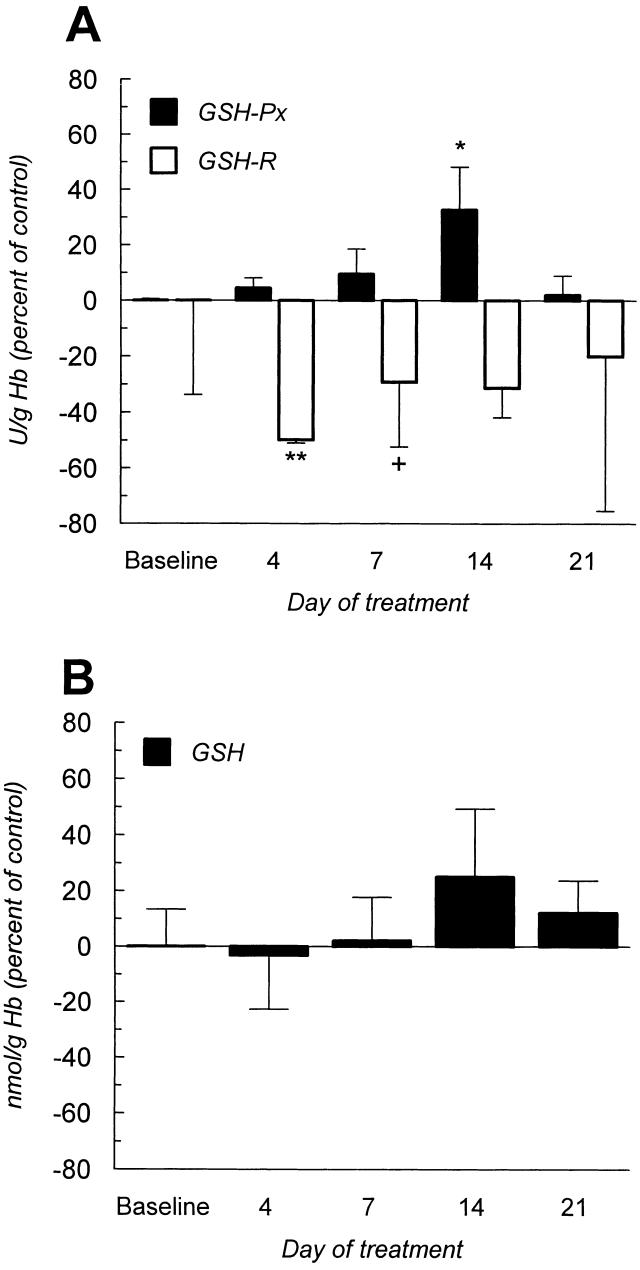

FIG. 4. Time course of changes in GSH-Px and GSH-R activities (A) and blood GSH contents (B) in ethanol-fed rats after 4, 7, 14, or 21 days of treatment. Values from control rats ($n = 4$) were set to 100% and values for ethanol-fed rats ($n = 4$ at each time point) represent percentage of values in control animals (mean ± SD). Data on GSH-Px and GSH-R activities and GSH contents are expressed in unit(U)/g Hb and nmol/g Hb, respectively. $*p < 0.05$, $+p = 0.073$, compared with controls (Student's *t*-test).

cantly decreased the intracellular GSH-R activity of the red blood cells, which reached ~50% of the control value after 4 days of treatment. The GSH-R activity displayed a linear profile [$Y = -48.334 + 1.37X$; $F = 4.46$, $p = 0.169$ (Spearman's coefficient: 0.831)], which differed markedly from that of GSH-Px throughout the experiments. It is likely, therefore, that chronic alcohol intoxication uncouples the enzymatic cycle of the glutathione system, resulting in a considerably decreased free radical-scavenging activity. Another possible explanation for these early responses is that the increased intracellular GSH content generated by reaction with excess free radicals may occupy most of the catalytic sites of GSH-R, thereby decreasing the GSH-R activity, and the increased GSH-R expression may restore the GSH-R activity.

Conflicting results have been published recently on the changes in activity and on the role of another antioxidant enzyme, catalase, in the protection against ethanol-induced cellular damage (7,23,31), but these alterations were influenced by the mode of ethanol treatment and also the type of tissue investigated. Chronic ethanol feeding, with a moderate dose of ethanol (~6 g/kg body weight), resulted in permanently reduced catalase activities. A slight decrease in catalase activity may reflect a minor role of the enzyme in detoxification processes when the H_2O_2 level is low (5).

The profile of the changes in the GSH content of the erythrocytes fully matched that of the TBARS content determined after chronic ethanol administration. A comparison of the TBARS contents of the erythrocytes and the plasma revealed a greater incidence of lipid peroxidation end-products in the latter, which presumably originate from organs such as the brain, heart, or liver (22,23,31,38) damaged by ethanol. It is interesting to note the characteristic shift in the maximum TBARS contents of plasma and erythrocytes (7 and 14 days, respectively), which may reflect the malfunctioning of liver antioxidant defense systems as the consequence of ethanol intoxication.

It is worthy of attention that changes were observed in all enzyme activities and in GSH content throughout the experiments, although the most pronounced suppression of antioxidant enzyme activities was established after 4 days of ethanol ingestion. These data may reflect early ROS-producing effects of chronic ethanol intoxication accompanied by the loss of detoxification mechanisms, whereas a progressive adaptation of the defense systems examined occurred in later phases of the experiments.

Conclusion

Our present findings provide evidence that chronic ethanol ingestion results in a correlative loss of explorative behavioral functions and the capacities of blood antioxidant, ROS-scavenging enzymes in rats. Moreover, the severity of behavioral changes and that of the deregulation of blood antioxidant defense systems closely correlates with the toxicity level of ethanol overload. It is noteworthy that chronic ethanol intoxication resulted in a rapid but transient decline in the activities of antioxidant enzymes, whereas only minor perturbations of the defense mechanisms were found in later phases of the experiment. We assume, therefore, that restoration of the ROS-scavenging mechanisms may play a critical role in behavioral adaptation to the toxic effects of ethanol. Furthermore, increases in $[Ca^{2+}]_i$ elicit a rapid generation of ROS (20), which may contribute directly to the behavioral deficits.

In conclusion, this model permits the direct study of early changes after chronic ethanol intoxication, and provides a novel model for their pharmacological prevention, based on comparative studies of animal behavior and ROS-scavenging enzyme activities at the periphery.

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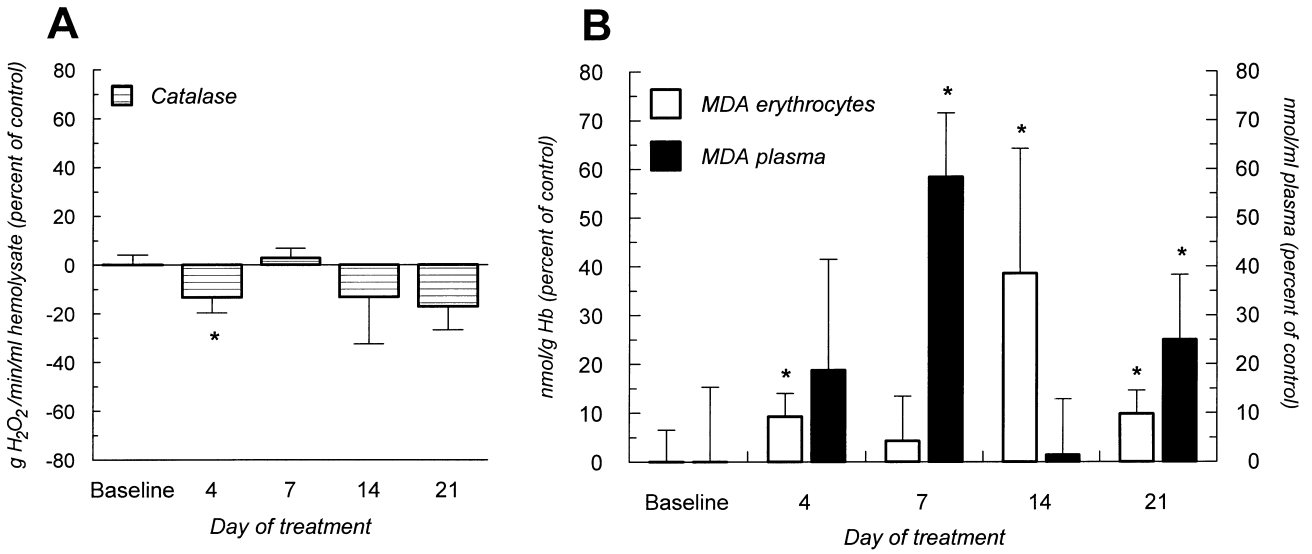

FIG. 5. Percent change (± SD) from control in catalase activity (A) and erythrocyte and plasma thiobarbituric acid reactive substance (TBARS) contents (B) after 4, 7, 14, or 21 days of chronic ethanol intoxication. Data on catalase activities and erythrocyte and plasma TBARS levels are expressed in g H_2O_2 hydrolyzed/min/ml hemolysate, nmol/g Hb, or nmol/ml plasma, respectively. Data for ethanol-fed rats are plotted as percent of their respective controls. Values represent mean ± SD. *$p < 0.05$, compared with controls (Student's *t*-test).

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