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Glutamate contributes to alcohol hepatotoxicity by enhancing oxidative stress in mitochondria

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Abstract Chronic alcohol intoxication is associated with increased oxidative stress. However, the mechanisms by which ethanol triggers an increase in the production of reactive oxygen species (ROS) and the role of mitochondria in the development of oxidative stress has been insufficiently studied. The biochemical and proteomic data obtained in the present work suggest that one of the main causes of an increase in ROS generation is enhanced oxidation of glutamate in response to long-term alcohol exposure. In the course of glutamate oxidation, liver mitochondria from alcoholic rats generated more superoxide anion and H_2O_2 than in the presence of other substrates and more than control organelles. In mitochondria from alcoholic rats, rates of H_2O_2 production and NAD reduction in the presence of glutamate were almost twice higher than in the control. The proteomic study revealed a higher content of glutamate dehydrogenase in liver mitochondria of rats subjected to chronic alcohol exposure. Simultaneously, the content of mitochondrial catalase decreased compared to control. Each of these factors stimulates the production of ROS in addition to ROS generated by the respiratory chain complex I. The results are consistent with the conclusion that glutamate contributes to alcohol hepatotoxicity by enhancing oxidative stress in mitochondria.

Keywords Chronic alcohol intoxication · Oxidative stress · Liver mitochondria · Reactive oxygen species · Glutamate dehydrogenase

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Introduction

Alcoholic liver disease is a worldwide health problem. The mechanisms that cause the progression of liver injury are complex. Although the pathogenesis of alcoholic liver disease involves changes in many systems and processes, a growing body of evidence indicates that the key elements are oxidative stress and mitochondrial dysfunction (Garcı̇'a-Ruiz et al. 2013; Louvet and Mathurin 2015; Mandrekar and Ambade 2012; Sid et al. 2013a; Teplova et al. 2010). Alcoholic intoxication causes not only substantial biochemical changes in the functioning of various mitochondrial enzymatic systems, such as the respiratory chain, the fatty acid oxidation pathway, and the urea cycle but also ultrastructural changes, mitochondrial DNA damage, and lipid accumulation (Auger et al. 2015; Cunningham and Bailey 2001; Grattagliano et al. 2011). Ethanol affects the mitochondrial functions, including respiration, ATP synthesis, and the production of reactive oxygen species (ROS) (Wu and Cederbaum 2009; Cederbaum et al. 2009; Mantena et al. 2008; Song et al. 2013; Sun et al. 2016; Yamashina et al. 2010). Recently, acute alcohol was shown to cause in vivo depolarization of hepatocellular mitochondria in a dose- and time-dependent fashion (Zhong et al. 2014). However, the reasons for the alcohol-induced increase in mitochondrial ROS production are not clear.

Recent data suggest that changes in the metabolism of pyridine nucleotides (PN) upon chronic alcohol intoxication

contribute to the development of various liver disorders. The oxidation of alcohol by alcohol dehydrogenase and acetaldehyde dehydrogenase considerably changes cytosolic and mitochondrial NADH/NAD ratios due to NADH accumulation (Cahill et al. 2002; Manzo-Avalos and Saavedra-Molina 2010; Zhong et al. 2014). Mitochondrial acetaldehyde dehydrogenase ALDH2 converts the toxic ethanol metabolite acetaldehyde into acetate and generates NADH in the mitochondrial matrix. Hence, fast NADH oxidation through the respiratory chain and NAD regeneration are important for the minimization of the toxic effect of ethanol and its metabolites. However, the data on the effect of ethanol consumption on the mitochondrial NADH oxidation are contradictory. Thus, acute alcohol exposure was reported to lead to a substantial decrease in the mitochondrial transmembrane potential and an increase in hepatic oxygen consumption (a phenomenon named SIAM, swift increase in alcohol metabolism) (Zhong et al. 2014). Similar changes in mitochondrial metabolism were detected upon liver adaptation to chronic ethanol consumption (Han et al. 2017). According to other data, prolonged ethanol consumption may break this adaptation since it causes a decrease in the activity of mitochondrial respiratory enzymes (Marcinkeviciute et al. 2000; Zelickson et al. 2011). Thus, incomplete fatty acid oxidation inherent in chronic alcohol intoxication promotes the inactivation of numerous enzymes (about 20% of mitochondrial proteins) by acetylation; their reactivation also requires high concentrations of oxidized NAD, which is deficient under the conditions of ethanol consumption (Nassir and Ibdah 2014). Presumably, the shift of the NADH/NAD balance to a more reduced state facilitates ROS production by damaged respiratory complexes and nonmitochondrial redox systems (Bailey and Cunningham 1998; Das and Vasudevan 2007; Kukielka et al. 1994). In mitochondria, several sites (respiratory complexes I, III, and mtCYP2E1) were reported to be responsible for increased ROS production upon chronic ethanol consumption (Leung and Nieto 2013; Bailey et al. 1999). However, the relation of mitochondrial NADH and ROS production through possible alcohol-induced changes in the activity of NAD/NADH-dependent dehydrogenases has not been established. It has been shown earlier that ethanol consumption causes a remarkable increase in the levels of glutamate, aspartate, and free ammonia, a glutamate oxidation product, in the liver (Cascales et al. 1985). Ethanol- and acetaldehyde-dependent inhibition of hepatocellular ureagenesis caused by relative closure of voltage dependent anion channels (VDAC) in the outer membrane may underlie these increases in glutamate, aspartate and ammonia (Holmuhamedov et al. 2012). Based on these data, it can be hypothesized that glutamate dehydrogenase may enhance oxidative stress induced by alcohol.

The aim of the present study was to elucidate the participation of glutamate dehydrogenase in the ROS production and systemic oxidative stress in liver mitochondria during chronic alcohol intoxication. Experiments were performed to examine the effect of long-term chronic ethanol consumption by rats on NAD reduction and ROS production linked to oxidation of substrates of NAD-dependent dehydrogenases. It was found that chronic ethanol consumption caused an increase in the activity of mitochondrial glutamate dehydrogenase, which appeared to be accompanied by a strong increase in ROS generation.

Materials and methods

Materials

Alamethicin, superoxide dismutase, antimycin A, rotenone, and 3,7-dihydro-2-methyl-6-(4-methoxyphenyl)imidazol[1,2-a]pyrazine-3-one (MCLA) were from Fluka (Sigma-Aldrich Corporation); carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), Amplex Red, NAD, NADH, menadione, and other reagents were from the Sigma-Aldrich Corporation (St. Louis, MO, USA).

Experimental model of chronic alcoholism

All manipulations with animals before the beginning of isolation of livers were performed in accordance with the Helsinki Declaration of 1975 (revised 1983) and national requirements for the care and use of laboratory animals. Manipulations were carried out by certified stuff of the Animal Department of the Institute of Theoretical and Experimental Biophysics (Russian Academy of Science) and approved by the Commission on Biomedical Ethics of ITEB RAS. We applied a widely used model of chronic rat alcogolization (Lang et al. 2004) as the closest to human alcoholism. Wistar male rats with a starting weight of 150 g were divided into two groups: a control group ($n = 15$), which received a food ration and drinking water, and an alcohol group ($n = 15$), in which animals were fed the same food and a 20% ethanol solution as the sole source of liquid for six to eight months. The addiction of the animals to alcohol was developed by gradually increasing its percent content in drinking water: 5% (days 1–3), 10% (days 4–6), 15% (days 7–10), and 20% (day 11– last day of experiment). During the study, the animals were kept in wire-mesh cages at a room temperature of 21–22 °C with a light/dark cycle of 12 h. Body weights were monitored weekly. Blood alcohol levels and liver weights were determined prior and after killing of animals, respectively, as physiological indicators of alcohol intoxication. The concentration of ethanol in blood serum was determined using enzymatic alcohol assay kit (Diagnostic Systems GmbH, Holzheim, Germany).

Liver samples were excised immediately after killing rats and homogenized in a 10-fold volume of 50 mM phosphate

buffer solution, pH 7.4. The homogenates were centrifuged at 10000 x *g* for about 60 min, and the resulting supernatants were stored at −80 °C until measurements of malondialdehyde (MDA) and reduced thiols. Rat liver mitochondria were isolated simultaneously from control and alcoholic rats by a standard differential centrifugation (Teplova et al. 2006). The mitochondria were washed twice in mannitol buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES-Tris, pH 7.4, potassium-free), resuspended in the same buffer to 60–80 mg protein/ml, and kept on ice. Protein levels in tissue and mitochondrial samples were determined by the Biuret method using BSA as a standard (Gornall et al. 1949).

Analysis of oxidative stress biomarkers

The level of liver free SH-groups was analyzed in liver homogenates by the method of Sedlak and Lindsay using the Ellman's reagent (Sedlak and Lindsay 1968). Malondialdehyde (MDA) was used as a biomarker of the lipid peroxidation level. The rates of MDA formation by liver homogenates were measured using thiobarbituric acid (Ohkawa et al. 1979).

Measurement of oxygen uptake

Isolated mitochondria (1.5 mg protein/ml) were incubated at 25 °C in a medium containing 120 mM KCl, 2 mM KH_2PO_4, and 10 mM HEPES (pH adjusted to 7.3 with Trizma base) supplemented with substrates of complex I (5 mM pyruvate and 5 mM malate) or complex II (5 mM succinate) in the presence of 2 μM rotenone. Oxygen uptake was measured with a Clark-type electrode using Oroboros Oxygraph-2 k (Austria). In order to assess V_3 and V_4 respiration rates, 500 μM ADP was added to resting mitochondria (V_2). The rate of uncoupled respiration (V_{DNP}) was measured in the presence of 50 μM 2,4-dinitrophenol (DNP).

Determination of pyridine nucleotide redox state in isolated mitochondria

Changes in mitochondrial functions were determined as described earlier (Teplova et al. 2006). The pyridine nucleotide (PN) redox state was determined by recording fluorescence of PN on a Hitachi F 7000 fluorimeter with excitation at 340 nm and emission at 460 nm. All parameters were measured in a standard KCl- medium containing 120 mM KCl, 2 mM KH_2PO_4, and 10 mM HEPES (pH adjusted to 7.3 with a few grains of TRIZMA base) with 5 mM substrates.

The activity of NAD-dependent dehydrogenases was estimated from the reduction of exogenous NAD in permeabilized mitochondria by recording PN fluorescence on a Hitachi F 7000 fluorimeter with excitation at 340 nm and emission at 460 nm. The experiments were carried out in the mannitol-sucrose medium (220 mM mannitol, 70 mM sucrose, and 10 mM HEPES (pH adjusted to 7.3) in the presence of alamethicin, rotenone, and cyanide with different respiration substrates. The details are given in the figure legends.

Estimation of ROS production by isolated mitochondria

The production of superoxide anion in a rat liver mitochondria was measured in standard KCl- medium supplemented with 1 mM EGTA and respiration substrates using the highly sensitive chemiluminescent probe MCLA (20 μM), which can also detect singlet oxygen (Kambayashi and Ogino 2003). The kinetics of the SOD-sensitive MCLA-derived chemiluminescence (MDCL) was recorded in 96-well plates (Greiner Bio-One, Germany) using a multi-plate reader (Infinite 200 Tecan, Austria), as described earlier (Nikiforova et al. 2014). H_2O_2 released from isolated mitochondria was measured in standard KCl medium supplemented with 20 μM Amplex Red and horseradish peroxidase (HRP) (4 U/ml) (Starkov 2010). In order to measure H_2O_2 production in permeabilized RLM, alamethicin (10 μg/ml) was added 5 min prior to HRP. Where indicated, media also contained NAD, coenzyme A, substrates of NAD-dependent dehydrogenases, and inhibitors of respiratory chain. Resorufin formation was traced for 40 min using a multiplate fluorimeter (Infinite 200 Tecan, Austria) at excitation and emission wavelengths 530 and 595 nm. For the quantitative assessment of H_2O_2 production, fluorescence was calibrated by a bollus of H_2O_2 at the end of measurements.

Two-dimensional gel electrophoresis of mitochondrial proteins and mass spectrometry protein identification

Mitochondrial proteins from control and ethanol-fed rats were diluted in a rehydration solution containing 7 M urea, 2% triton X-100, 5% mercaptoethanol, and 2% ampholytes (pH 3.5–10). The polyacrylamide gel columns with the proteins separated by isoelectric focusing (IEF) were applied as a start zone for separation in the second direction. Two-dimensional IEF/SDS-PAGE was performed using a linear 7.5–0% gradient of acrylamide concentration in the presence of 0.1% SDS in a Helicon (Russia) vertical electrophoretic cell, as described earlier (Kovalyov et al. 1995). Protein visualization by Coomassie Blue R-250 and silver nitrate staining as well as the analysis of two-dimensional (2D) electrophoregrams were performed as described earlier (Kovalyov et al. 1995; Kovalev et al. 2006) with minor modifications (Kovalyova et al. 2009). Molecular masses of proteins in the electrophoretic fractions were determined using the kits of highly purified recombinant proteins SM0671 (10–170 kDa) (Fermentas, USA).

Protein spots with altered characteristics, found by the image analysis, were excised from gels and subjected to matrix-

assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The tryptic hydrolysis of single protein fractions followed by an analysis of tryptic peptides using MALDI TOF MS and MS/MS were used for identification of the proteins as described earlier (Govorun et al. 2003; Kovalev et al. 2006; Shevchenko et al. 1996). Mass-spectra were obtained on a MALDI-TOF-mass-spectrometer Reflex III (Bruker, USA) with UV-laser (336 nm) in the regime of positive ions in the range of 500–8000 Da and calibrated in accordance with the known peaks of trypsin autolysis. Proteins were identified using the Mascot software, option Peptide Fingerprint (Matrix Science, USA) by matching experimental masses with the masses of proteins listed in the NCBI Protein and SwissProt/treMBL databases.

The densitometry of 2D electrophoregrams and/or their individual fragments (rectangles) was performed after scanning (Epson Expression 1680 scanner). The computer handling of the resultant densitometry images of protein fractions was performed using the Melanie Image Master software, versions 6 and 7 (Genebio, Switzerland).

Statistical analysis

The data given represent the means ± standard error of means (SEM) from 5 to 7 animals per treatment group or are the typical traces of four to five identical experiments with the use of different mitochondrial preparations. The statistical significance was estimated by the Student's t-test with $p < 0.05$ as the criterion of significance.

Results

Signs of oxidative stress and mitochondrial dysfunction in the alcoholic liver

A quantitative estimation of oxidative stress biomarkers, such as MDA and free SH-groups in liver tissues, was performed. After seven months of alcohol consumption, rats showed a high content of alcohol in blood (165 ± 24 mg/dl), a smaller body weight (460 ± 25 g versus 570 ± 30 g in control), and a greater liver weight (14.3 ± 0.8 g versus 11.8 ± 0.6 g in control). The rate of MDA formation increased by 15% (755.8 ± 14.1 versus 603.7 ± 12.5 μmol/h/g of liver in control), and the concentration of free SH-groups decreased by 35% in alcoholic liver tissues (5.42 ± 0.24 versus 8.34 ± 0.35 μmol/g of liver in control). All changes were statistically significant. Earlier in the same model, we found that chronic alcohol intoxication increased the level of triglycerides in the liver from 9.6 ± 1.5 to 42.3 ± 5.2 μg/mg protein and in the blood serum from 102.1 ± 10.5 to 160.3 ± 21.0 mg/dL, which indicates an alcohol-induced disturbance of the catabolism of fats (Shubina et al. 2013).

In accordance with earlier observations (Marcinkeviciute et al. 2000), the respiratory activity (V_2, V_3, V_4, and the rate of uncoupled respiration (V_{DNP})) was considerably depressed in alcoholic mitochondria in the presence of substrates, which feed both complex I (glutamate plus malate) and II (succinate in the presence of rotenone) (Figs. 1a and b). These data indicate an enhancement of oxidative stress, an increase in the intensity of peroxidation processes, and the development of mitochondrial dysfunction in the liver of rats that received alcohol for long time.

Effect of long-term alcohol consumption on ROS production by liver mitochondria

To clarify the role of mitochondria in the development of oxidative stress in chronic alcohol intoxication, we compared the ability of liver mitochondria from control and alcoholic rats to generate ROS in the presence of different respiratory substrates, inhibitors of complex I and the redox cycler menadione. Long-term alcohol consumption caused an increase in H_2O_2 production by rat liver mitochondria (Fig. 2a). The higher rates were observed in the presence of glutamate plus malate than in the presence of succinate plus rotenone or without added substrate. The superoxide anion production in the presence of glutamate and malate was also higher in liver mitochondria of alcoholic animals (Fig. 2c–d). Menadione stimulated the superoxide production with a rate higher than in control mitochondria. Menadione bypasses the rotenone
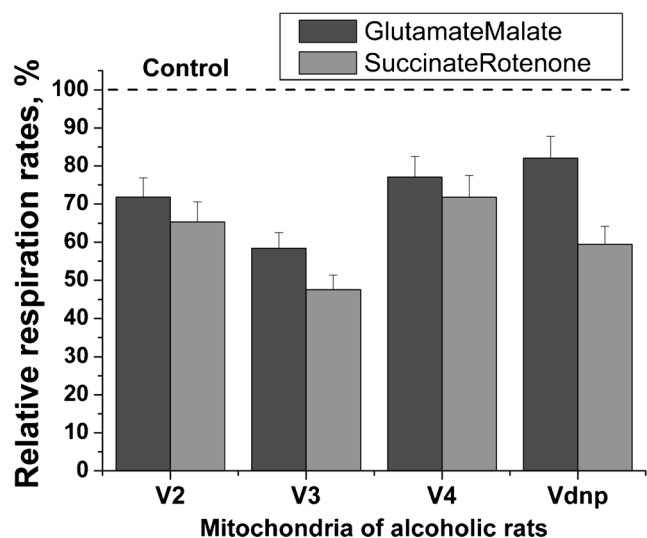

Fig. 1 Oxygen consumption by control and alcoholic liver mitochondria, oxidizing glutamate plus malate and succinate in the presence of rotenone. Experimental details are given in Materials and methods. Rates of V_2, V_3, V_4, and V_{DNP} respiration in control mitochondria are taken as 100% and correspond to 8.5 ± 0.3, 42.8 ± 2.1, 9.5 ± 0.5, and 45 ± 3.2 ng-atom O·min^{-1}·mg protein^{-1} in the presence of glutamate plus malate and 14.9 ± 0.7, 80.3 ± 4.1, 19.2 ± 0.9, and 135.4 ± 6.7 ng-atom O·min^{-1}·mg protein^{-1} in the presence of succinate plus rotenone. All differences between control and alcoholic mitochondria are significant ($p < 0.05$)

block in complex I and provides the transfer of electrons from NAD(P)H to cytochrome *c* and directly to molecular oxygen, leading to ROS generation (Criddle et al. 2006; Moore et al. 1986). The specific inhibitor of complex I rotenone, which acts at the quinone-binding site (Brand 2016), as well as the nonspecific inhibitor of complex I NEM, which, presumably, disturbs the flavine-binding site (Gavrikova and Vinogradov 1999; Gostimskaya et al. 2006), activated the superoxide production, which was also stronger in mitochondria of alcoholic rats. The most pronounced differences in superoxide anion generation were observed in the presence of the complex I inhibitors (rotenone, NEM) but not complex III inhibitor (antimycin A). These data suggest that complex I contributes to increased ROS production in liver mitochondria from alcoholic rats. Another reason for the faster ROS production in

these mitochondria may be the contribution of NAD-dependent dehydrogenases.

Effect of long-term alcohol consumption on reduced pyridine nucleotide levels in rat liver mitochondria

The level of endogenous NAD(P)H in liver mitochondria from alcoholic rats was about 15–25% higher than in mitochondria from control animals (Fig. 3a). Panels B, C and D in Fig. 3 show changes of NAD(P)H fluorescence on addition of rotenone, substrates of NAD-dependent dehydrogenases and menadione. Rotenone, which completely blocks NADH oxidation by complex I, increased the reduction of endogenous PN. Menadione induced complete oxidation of NAD(P)H in the presence of all substrates used: glutamate plus malate (B),
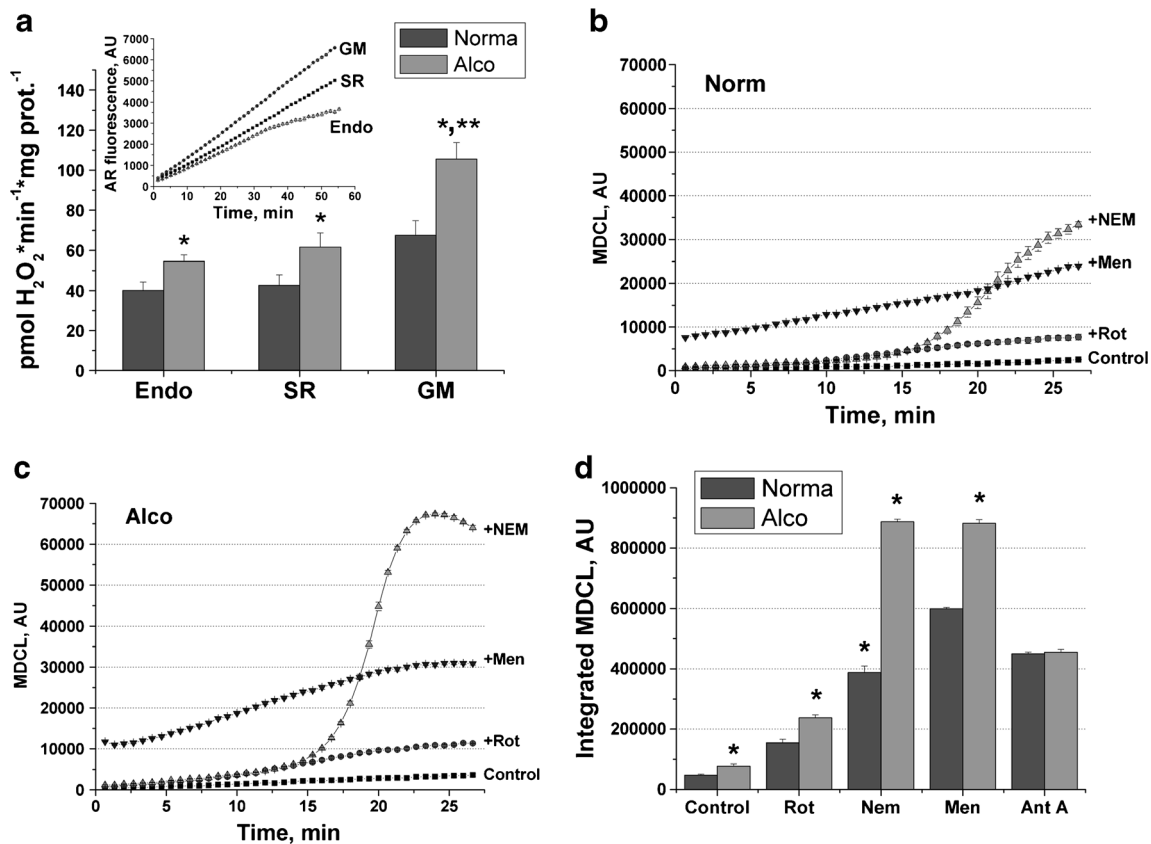

Fig. 2 Hydrogen peroxide and superoxide anion production by intact liver mitochondria from control and alcoholic rats in the presence of respiratory substrates and respiratory chain inhibitors. Panel **a**. Original traces of a representative experiment with alcoholic rat liver mitochondria (insert, AR – *Amplex Red*) and the rates of substrate-dependent H_2O_2 production are shown. Liver mitochondria from control and alcoholic rats (0.4 mg protein/ml) were added to standard medium, which contained 1 mM EGTA, 20 μM Amplex Red, 4 U/ml HRP and, where indicated, 5 mM glutamate plus 5 mM malate (GM), 5 mM succinate plus 2 μg/ml rotenone (SR). *Asterisks* show the significant difference between control and alcoholic mitochondria; *double asterisks* indicate significant difference between glutamate- and succinate-supplemented alcoholic mitochondria ($n = 15$). Panels **b** and **c** show representative traces of

superoxide anion production simultaneously measured in liver mitochondria from control (panel **b**) and alcoholic rats (panel **c**). Mitochondria (0.3 mg protein/ml) were added to standard medium supplemented with 5 mM glutamate, 5 mM malate and 20 μM MCLA. Where indicated, medium also contained 20 μM NEM (+NEM), 20 μM menadione (+Men), and 1 μg/ml rotenone (+Rot). Data on traces in panels A–C are the means ± S.E.M. ($n = 3$). Panel **d**. Integrated MCLA-derived chemiluminescence (MDCL) measured for 25 min in the same samples and in antimycin A (1 μg/ml)-containing samples is presented. The SOD-insensitive part of MDCL was subtracted from total MDCL. Asterisks show the significant difference between control and alcoholic mitochondria
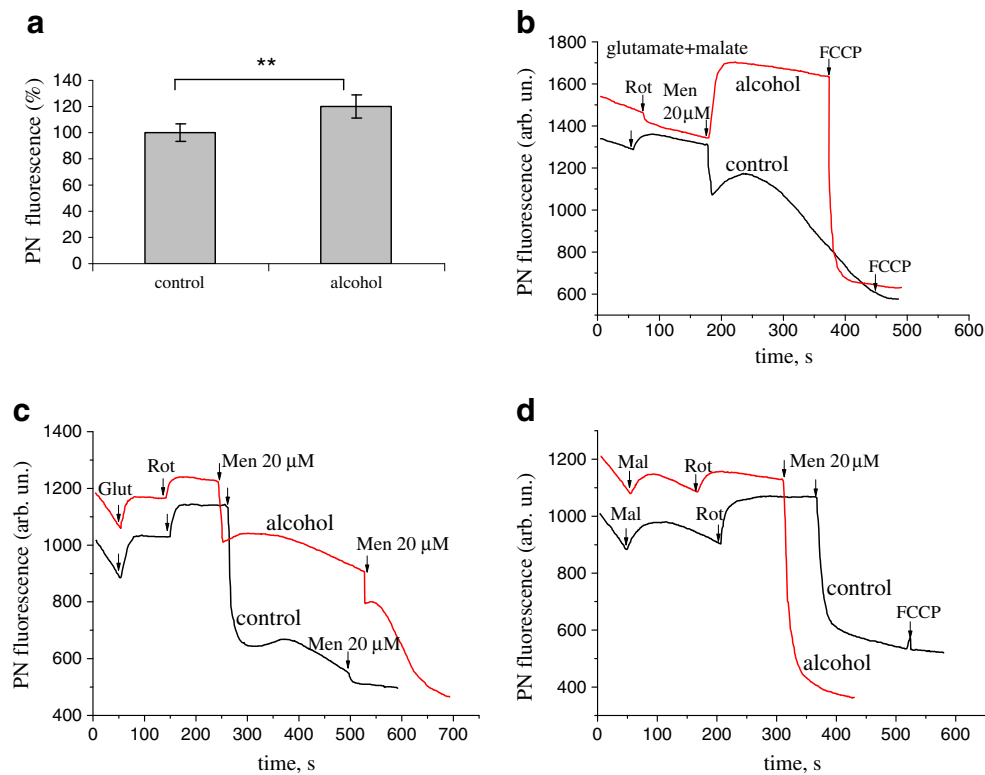
Fig. 3 Effect of substrates, rotenone and redox-cycling menadione on the redox state of PN in liver mitochondria from normal and alcoholic rats. Mitochondria (1 mg protein/ml) were added to standard medium without substrates (**a**, **c**, and **d**) or to medium supplemented with 5 mM glutamate plus 5 mM malate (**b**). Where indicated, 5 mM glutamate, 5 mM malate, 2 μg/ml rotenone, menadione and 0.5 μM FCCP were added. Traces are representative of at least four similar experiments

glutamate (C), and malate (D) in control mitochondria. In contrast, in mitochondria of alcoholic rats in the presence of glutamate plus malate, menadione increased PN reduction (Fig. 3b). Glutamate alone but not malate alone was also capable of maintaining the NAD(P)H level in the presence of menadione (Figs. 3c and d). To oxidize NAD(P)H fully in mitochondria from alcoholic livers, menadione concentrations higher than required for control mitochondria were necessary. Thus, the glutamate oxidation made a major contribution to increased reduction of NAD(P)H in liver mitochondria of alcoholic rats.

In subsequent experiments, the reduction of NAD in permeabilized liver mitochondria in the presence of different NAD-dependent substrates was analyzed (Fig. 4). Mitochondria were permeabilized by the freezing-thawing and the addition of pore-forming alamethicin. Under these conditions, NAD penetrated across mitochondrial membranes and was reduced by matrix dehydrogenases. Inserts A and B of Fig. 4 show the experimental design (A) and original traces of NAD reduction in the presence of glutamate in liver mitochondria from control and alcoholic rats (B). The main panel shows the initial rates of NAD reduction in the presence of malate, pyruvate, α-ketoglutarate, and glutamate. These data indicate that, in comparison with other substrates, glutamate provides the highest rate of NAD reduction in both
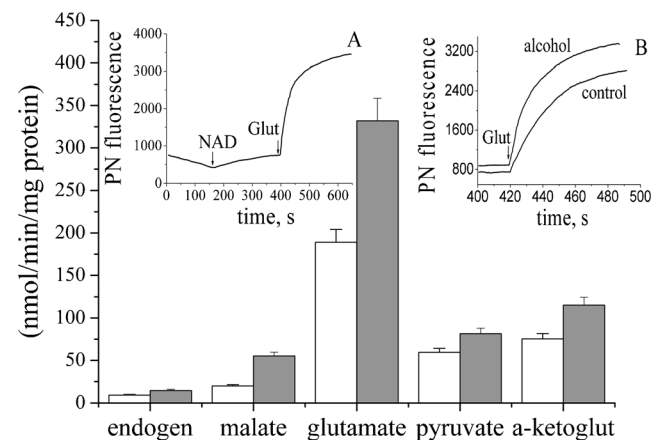

Fig. 4 NAD reduction in permeabilized liver mitochondria from normal and alcoholic rats upon oxidation of NAD-dependent substrates. Liver mitochondria (1 mg/ml) from control and alcoholic rats were permeabilized by one freezing-thawing cycle and incubated in the presence of pore-forming alamethicin (10 μg/ml). Inserts A and B show the experimental design (**a**) and original traces of NAD reduction in the presence of 5 mM glutamate and 500 μM NAD in liver mitochondria from control and alcoholic rats (**b**). The main panel shows the calculated rates of NAD reduction. In the presence of pyruvate and α-ketoglutarate, the medium also contained 500 μM CoA. The values are means ± S.E.M. of four independent experiments
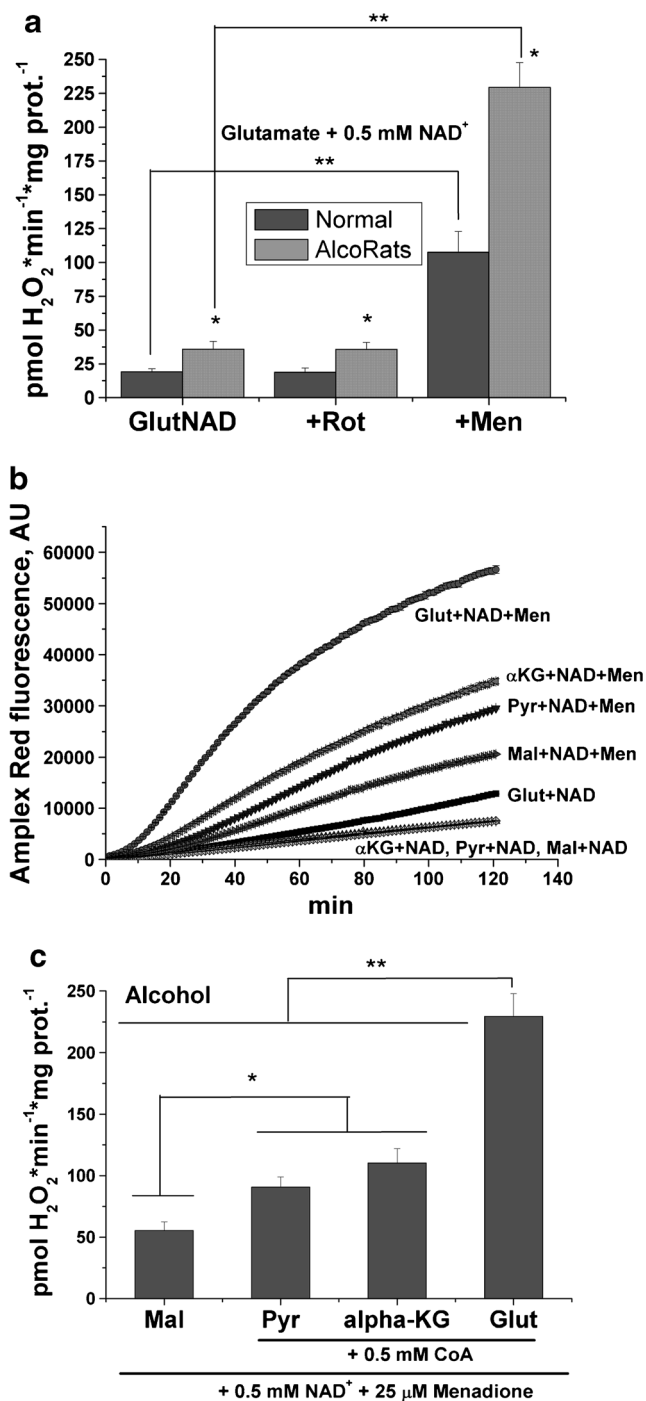
a

b

c

alamethicin. In the presence of the substrates of glutamate dehydrogenase (NAD and glutamate), H_2O_2 production by permeabilized mitochondria from alcoholic rats was twice that of mitochondria of control rats (Fig. 5a). This difference persisted in the presence of rotenone and strongly increased in the presence of menadione. Figures 5b and c show that the H_2O_2 production in the presence of glutamate (+/− menadione) was much higher than in the presence of the substrates of other NAD-dependent dehydrogenases. These data indicate that in alcoholic intoxication the major part of mitochondrial ROS generation is due to the higher activity of glutamate dehydrogenase.

Effect of chronic alcohol consumption on the mitochondrial proteome

The chronic alcohol consumption leads to a change in the expression of a number of mitochondrial proteins. Therefore, the higher glutamate dehydrogenase activity in alcoholic mitochondria may be caused by an increase in its content. In subsequent experiments, the effect of chronic alcohol consumption on the level of the glutamate dehydrogenase as well as antioxidant enzymes in the whole mitochondrial proteome was analyzed. Two-dimensional gel electrophoresis of liver mitochondria from control and alcoholic rats and analysis of changes in the mitochondrial proteome induced by chronic ethanol consumption revealed a strong increase in the amount of mitochondrial glutamate dehydrogenase (Fig. 6). In addition, catalase decreased. Details of mass spectrometric identification of protein fractions and characteristics of spots in 2D–gels are given in Table 1 (N 1–3). Three-dimensional densitometry of the zones of glutamate dehydrogenase and catalase in the 2D gel images confirmed the increases of glutamate dehydrogenase and decrease of catalase (Fig. 7).

As seen from Figs. 6 and 7, the content of glutamate dehydrogenase precursors increased in alcoholic mitochondria about threefold. Furthermore, proteomic studies revealed two fractions of catalase, acidic (2) and alkaline (3), the latter

mitochondrial samples. However, in mitochondria of alcoholic rats, this rate was almost twice higher than in control mitochondrial samples.

Contribution of NAD-dependent dehydrogenases to ROS production in alcoholic intoxication

The contribution of NAD-dependent dehydrogenases to ROS production was studied under the same conditions in mitochondria permeabilized by the freezing-thawing and
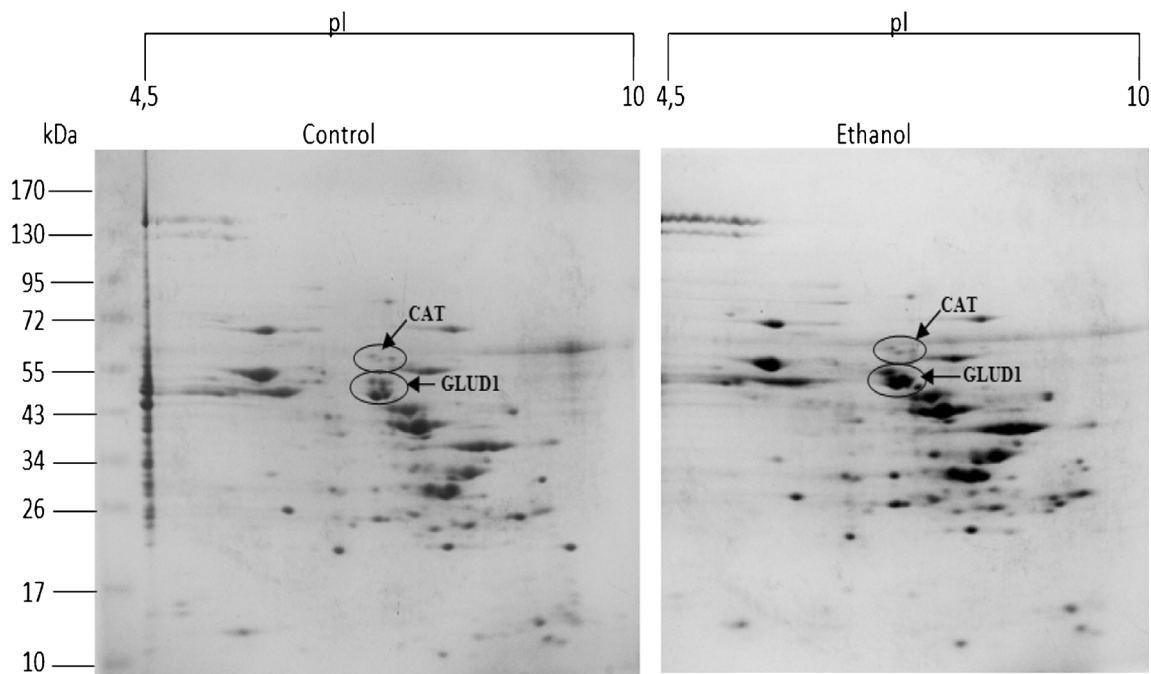
Fig. 6 Two-dimensional gel electrophoresis of liver mitochondria from control and alcoholic rats. Representative high resolution separation of mitochondrial proteins from a control and an alcoholic rats by two-dimensional IEF/SDS-PAGE. Ellipses depict the locations of glutamate dehydrogenase (*GLUD1*) and catalase (*CAT*)

in normal rat liver mitochondria being significantly larger. In alcoholic rat liver mitochondria, the alkaline catalase fraction is distinctly reduced.

Overall, the data obtained are consistent with the conclusion that increased glutamate dehydrogenase and decreased catalase contributed to ROS overproduction in liver mitochondria of alcoholic rats.

Discussion

The results obtained indicate that chronic ethanol consumption caused an increase in the activity of mitochondrial glutamate dehydrogenase, which was related to an increase in protein expression of the mitochondrial glutamate dehydrogenase. In the presence of glutamate, mitochondria from

alcoholic rats generated more superoxide anion and H_2O_2 than in the presence of other substrates and more than control mitochondria. ROS generation after alcohol was to a large measure associated with the high activity of glutamate dehydrogenase. In addition, a lower content of mitochondrial catalase was found compared to non-alcoholic rats. Both factors appeared to promote disorders in matrix systems of NADH reduction and oxidation, excessive ROS production, and systemic oxidative stress, which are the distinguishing features of chronic alcohol intoxication.

The alcohol-induced activation of glutamate dehydrogenase, in particular in the liver and leukocytes, has been reported earlier (Cascales et al. 1985; Kravos and Malesic 2010). The activity of mitochondrial glutamate dehydrogenase was shown to rise after both chronic ethanol and acetaldehyde treatment (Cascales et al. 1985). Interestingly, alcohol was also found to increase the glutamate in blood, and blood

**Table 1** Mass spectrometric (MALDI-TOF MS and MS/MS) identification of protein fractions

N	Protein name (gene symbols)	Accession number*	S/M/C **	Mm/pI (exp.)	Mm/pI (calc.)***
1	glutamate dehydrogenase, (*GLUD1*)	6,980,956	389/40/60	55.5/7.50	55.2/7.02
2	Catalase (*CAT*)	6,978,607	352/41/77	60.0/7.65	59.7/7.07
3	Catalase (*CAT*)	6,978,607	511/46/81	60.0/7.70	59.7/7.07

*The accession number indicates the registration number for the NCBI protein

**S/M/C – protein score/match peptide/coverage % (indicators of identification)

***Mm/pI (calc.) – calculated estimates made from the amino acid sequence data after the subtraction of the signal peptide using the program ExPASy Compute pI / Mw tool

Fig. 7 Zones of glutamate dehydrogenase and catalase in 2D gel images (**a**, **c**) and 3D computer visualization of densitometry (**b**, **d**). Numbered *arrows* correspond to those in Table 1
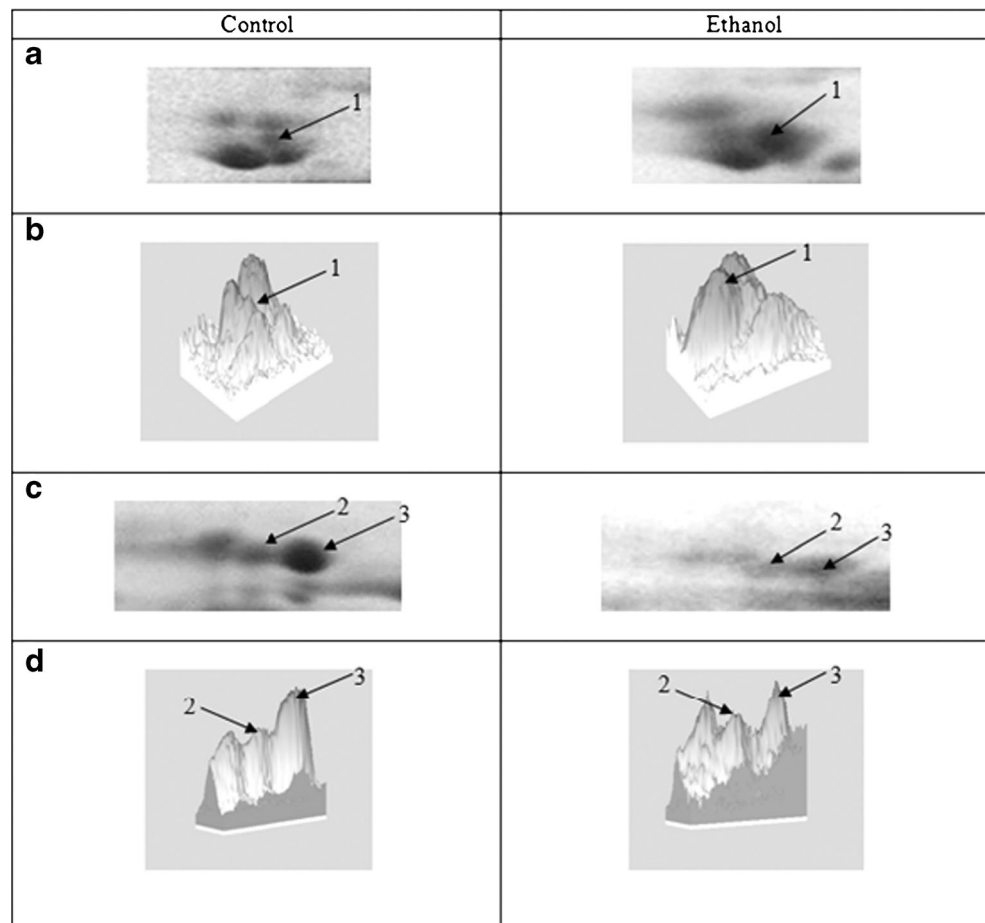
glutamate has been suggested as a biological marker of alcohol dependence (Tominaga et al. 1993; Walter et al. 2006). These data indicate a special role of glutamate in alcohol intoxication. It has been shown earlier that ethanol consumption causes a marked increase in the level of not only glutamate but also aspartate and free ammonia in the liver as well as a decrease in pyruvate and 2-oxoglutarate concentrations (Cascales et al. 1985). Ammonia and 2-oxoglutarate are the direct metabolites of glutamate oxidation, while aspartate can form in transamination reactions. Accumulation of these metabolites may be related to the more recently described inhibition of hepatocellular ureagenesis by ethanol and acetaldehyde (Holmuhamedov et al. 2012). Importantly, ammonia strongly activates H_2O_2 production in mitochondria, presumably affecting the interaction of NAD(H) and lipoamide dehydrogenase (Grivennikova et al. 2008). These data agree well with our results showing a considerable contribution of glutamate oxidation to oxidative stress. Indeed, generation of ammonia by glutamate dehydrogenase can be responsible for the higher rates of ROS generation in the presence of glutamate. Consequently, glutamate can stimulate ROS production simultaneously by two pathways, namely, NAD reduction and ammonium formation. Against the background of the

diminished activity of respiratory complex I (Bailey et al. 1999; Hoek et al. 2002; Manzo-Avalos and Saavedra-Molina 2010; Sun et al. 2016), the high glutamate dehydrogenase activity causes the overreduction of NAD, which, in turn, further stimulates ROS production.

The overproduction of NADH, also referred to as reductive stress (Gores et al. 1989), is inherent in many pathological and prepathological states. Along with alcohol intoxication, these are diabetes, ageing and others disorders (Gomes et al. 2013; Yan 2014). These states are as a rule associated with hypoxia or pseudohypoxia. These disorders are caused by the dysfunction of complex I and the increased NADH/NAD ratio. They are accompanied by the activation of fatty acid and triacylglycerol synthesis (He et al. 2013). Earlier, in our alcoholic model we found increased levels of triglycerides in the liver and blood serum, which confirms the disturbance of the catabolism of fats (Shubina et al. 2013). Mitochondrial dysfunction is assumed to be a main factor leading to obesity, a characteristic feature of chronic alcohol abuse. In this connection, glutamate itself induces the obesity syndrome with the attendant accumulation of triglyceride and cholesterol in blood, liver steatosis, and the formation of 4-hydroxynonenal (Sasaki et al. 2011). Besides neuroexcitotoxicity and obesity,

the chronic administration of glutamate leads to oxidative stress in hepatic and cardiac tissues in experimental animals, which can be attenuated by antioxidants (Kumar and Bhandari 2013). Based on these data, the toxic action of glutamate in alcoholism may also promote liver steatosis.

Respiration regenerates NAD, a rate-limiting substrate in alcohol/acetaldehyde metabolism. Other mechanisms of NAD regeneration are also possible, among them the shunting of complex I (inhibited or damaged) by quinones. Quinones can bypass complex I and transfer electrons to cytochrome c, activate H^+ pumps of complex IV, and thus create $\Delta\Psi m$ in deenergized mitochondria (Dedukhova et al. 1986; Dedukhova and Mokhova 1987; Fedotcheva et al. 2010). In turn, $\Delta\Psi m$ can maintain the NADH-dependent reduction of NADP in transhydrogenase reaction (Eytan et al. 1990). In agreement, menadione induced overreduction of NAD(P) in liver mitochondria from alcoholic rats in the presence of glutamate and rotenone, maintaining NADH in a reduced state. In order to decrease NADH in these mitochondria, menadione concentrations higher than in the control were necessary. Thus, glutamate contributes to alcohol hepatotoxicity, enhancing both oxidative and reductive stress in mitochondria.

Two-dimensional electrophoresis in combination with mass spectrometry is widely used for the identification of changes in protein expression (Uto et al. 2010). Proteomic changes in the liver have been investigated earlier on animal models of acute and chronic alcohol treatment. The studies by Cunningham and colleagues demonstrated that chronic alcohol consumption induces a decrease in the synthesis of 13 mitochondrial proteins that are components of complexes I, III, IV, and V (Coleman and Cunningham 1990; Coleman and Cunningham 1991). Alcohol exposure also changes the content of enzymes involved in the oxidation of fatty acids and oxidative phosphorylation (Venkatraman et al. 2004). Here, a proteomic approach was used to estimate alcohol-induced changes in the content of mitochondrial proteins, particularly glutamate dehydrogenase and antioxidant enzymes. The data obtained showed that alcohol increased the amount of the mitochondrial glutamate dehydrogenase and decreased the content of mitochondrial catalase.

Earlier it was shown that chronic alcohol consumption significantly decreased the level of mitochondrial complexes I, III, IV, and V, as well as the level of hepatic PGC1α, NRF1, and TFAM. It was assumed that the down regulation of transcription factors is the key mechanism underlying the chronic alcohol consumption-induced suppression of mitochondrial biogenesis and respiration (Sun et al. 2016). Nevertheless, in some cases, alcohol feeding caused an enhancement in glutamate/malate- and glycerol-3-phosphate-driven respiration and an increase in the expression of mitochondrial glycerol phosphate dehydrogenase (Han et al. 2017). It was suggested that liver mitochondria are remodeled in response to alcohol feeding. Our data show that the increase in GDH

protein level and the decrease in the respiratory activity are a consequence of chronic alcohol consumption. Probably, the alcohol-induced ROS production can activate signaling pathways involved in the mitochondrial remodeling. Thus, it can induce the activation of AMP-activated protein kinase, which controls the mitochondrial functions under conditions of oxidative stress induced by alcohol (Sid et al. 2013b).

The decrease in the catalase level may be an adaptation to chronic ethanol exposure since catalase, in contrast to GSH-related systems, removes hydrogen peroxide without oxidation of NAD(P)H and hence is useless for the alleviation of the reductive stress. The literature data on the effect of alcohol on the activity of antioxidant enzymes in liver mitochondria (MnSOD, glutathione peroxidase, glutathione reductase) are controversial. For instance, the studies of Tang et al. (2012) and Zhang et al. (2015) showed that the activity of antioxidant enzymes decreases. In contrast, Zeng et al. (2008), Larosche et al. (2009), and Vidyashankar et al. (2012) showed no decrease and even found an increase in the activity. The activity of catalase, however, decreased in the majority of cases (Zeng et al. 2008; Vidyashankar et al. 2012).

Thus, the results obtained in the present work indicate that chronic ethanol consumption causes an increase in the activity of glutamate dehydrogenase, which is related to the increase in the amount of this enzyme in liver mitochondria. Together with the lower content of mitochondrial catalase, these factors promote disorders in matrix systems of NADH reduction and oxidation, excessive ROS production, and systemic oxidative stress, which are the distinguishing features of alcohol intoxication.

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