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ALCOHOL HANGOVER INDUCES MITOCHONDRIAL DYSFUNCTION AND FREE RADICAL PRODUCTION IN MOUSE CEREBELLUM

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Abstract—Alcohol hangover (AH) is defined as the temporary state after alcohol binge-like drinking, starting when ethanol (EtOH) is absent in plasma. Previous data indicate that AH induces mitochondrial dysfunction and free radical production in mouse brain cortex. The aim of this work was to study mitochondrial function and reactive oxygen species production in mouse cerebellum at the onset of AH. Male mice received a single i.p. injection of EtOH (3.8 g/kg BW) or saline solution. Mitochondrial function was evaluated 6 h after injection (AH onset). At the onset of AH, malate-glutamate and succinate-supported state 4 oxygen uptake was 2.3 and 1.9-fold increased leading to a reduction in respiratory control of 55% and 48% respectively, as compared with controls. Decreases of 38% and 16% were found in Complex I–III and IV activities. Complex II–III activity was not affected by AH. Mitochondrial membrane potential and mitochondrial permeability changes were evaluated by flow cytometry. Mitochondrial membrane potential and permeability were decreased by AH in cerebellum mitochondria. Together with this, AH induced a 25% increase in superoxide anion and a 92% increase in hydrogen peroxide production in cerebellum mitochondria. Related to nitric oxide (NO) metabolism, neuronal nitric oxide synthase (nNOS) protein expression was 52% decreased by the hangover condition compared with control group. No differences were found in cerebellum NO production between control and treated mice. The present work demonstrates that the physiopathological state of AH involves mitochondrial dysfunction in mouse cerebellum showing the long-lasting effects of acute EtOH exposure in the central nervous system.

Key words: Alcohol hangover, Mitochondrial dysfunction, Free radicals, Oxidative stress.

INTRODUCTION

Excessive alcohol consumption has a variety of serious consequences on health. The different effects are widespread, altering numerous physiological, endocrine and behavioral functions. A particular state after binge-like drinking is defined as alcohol hangover (AH). The latter is described in humans as a physiological state which involves the unpleasant next-day effect following an evening of excessive alcohol consumption (Verster, 2009). Hangover begins when ethanol is absent in plasma and is characterized by a cluster of physical and psychological symptoms which include headaches, nausea, diarrhea, fatigue and tremors combined with decreased occupational, cognitive and/or visuospatial skills (Kim et al., 2003a,b; Howland et al., 2008). For experimental animals, AH provokes hypo-activity (Doremus-Fitzwater and Spear, 2007), fluctuations in body temperature, anxiety-like behavior (Zhang et al., 2007) and reduced wheel running activity (Brasser and Spear, 2002). These observations are in accordance to our previous data in which we demonstrated that hangover induced serious motor and affective impairment which persist several hours after acute ethanol exposure (Karadayian and Cutrera, 2013; Karadayian et al., 2013).

It was widely established that ethanol metabolism generates reactive oxygen species (ROS) which cause oxidative stress and lipid peroxidation in the brain (Calabrese et al., 1998; Comporti et al., 2010). Particularly, chronic alcohol exposure induces liver mitochondrial DNA damage mainly affecting respiratory complexes' activities (Cahill and Cunningham, 2000). In addition, previous researches indicate that alcohol could induce ROS production in the brain, heart and lung independently of its metabolism (Roberts et al., 1994). Interestingly, changes in mitochondrial nitric oxide (NO) metabolism were associated to memory and learning disabilities due to acute ethanol consumption (Chandler et al., 1994). The mentioned scientific reports evaluated

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Abbreviations: AH, alcohol hangover; CsA, cyclosporin A; $DiOC_6$, 3,3″-dihexyloxacarbocyanine iodide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; eNOS, endothelial nitric oxide synthase; EtOH, ethanol; FCCP, carbonyl cyanide p-trifluorome thoxyphenylhydrazone; GABA, gamma-Aminobutyric acid; H_2O_2, hydrogen peroxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesul fonic acid; HRP, horseradish peroxidase; iNOS, inducible nitric oxide synthase; MPT, mitochondrial permeability transition; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; $O_2^{\cdot-}$, superoxide anion; RCR, respiratory control ratio; ROS, reactive oxygen species; SOD, superoxide dismutase; SSC, side scatter; VDAC, voltage-dependent anion channel.

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the effects of chronic or acute alcohol exposure when alcohol still persisted in blood. Jung and Metzger (2010) determined that alcohol withdrawal provokes neuronal integrity damage being the brain tissue the most affected area. In this sense, we previously demonstrated mitochondrial dysfunction, oxygen free radical production and NO metabolism alterations at the onset of AH in mouse brain cortex 6 h after acute ethanol exposure associating this to a reduction in motor performance (Bustamante et al., 2012; Karadayian et al., 2014). The motor disturbances due to hangover observed in our previous researches could be attributed not only to a possible mitochondrial dysfunction in the cerebral cortex but also to an impaired functionality of the cerebellum, area that integrates sensory and motor pathways. Taking all together into account, the aim of this work was to study mitochondrial function and free radical production in mouse cerebellum at the onset of AH.

EXPERIMENTAL PROCEDURES

Materials

ADP, antimycin, bromophenol blue, $CaCl_2$, catalase, citochrome c, dithiothreitol, DTT, EDTA, EGTA, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), free fatty acid BSA, Folin reagent, glutamate, Hepes, hydrogen peroxide (H_2O_2), KH_2PO_4, horseradish peroxidase (HRP), KCl, K_2HPO_4, KCN, L-arginine, malate, mannitol, $MgCl_2$, NaH_2PO_4, Na_2HPO_4, NaN_3, NADH, NADPH, oxyhemoglobin, scopoletin, SDS, succinate, sucrose, superoxide dismutase (SOD), Tween were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). The probes 3,3″-dihexyloxacarbocyanine iodide ($DiOC_6$) and MitoSOX together with neuronal nitric oxide synthase (nNOS) and inducible NOS (iNOS) rabbit antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Goat voltage-dependent anion channel (VDAC) antibody was obtained from Molecular Probes Inc, USA. APS, 2-mercaptoethanol, bisacrylamide, Laemmli Buffer, TEMED were acquired from Bio Rad (Laboratories Inc, Research Foundation, Oklahoma, USA). Other reagents were of analytical grade.

Animals

Swiss mice (*Mus musculus*) weighing 30 g housed in a soundproof room, with humidity and-controlled temperature (22 ± 2 °C) with a 12:12-hour light: dark cycle photoperiod (lights on 7:00 am), fed standard rat chow and tap water "*ad libitum*" were used. Animal handling and treatment, as well as all experimental procedures were reviewed in accordance with the guidelines of the National Institutes of Health (USA), and with the 6344/96 regulation of the Argentinean National Drug Food and Medical Technology Administration (ANMAT). Moreover, the present study had the legal ethical accreditation from Ethics Committee for Laboratory Animal Handling of the School of Pharmacy and Biochemistry from Universidad de Buenos Aires where the protocol was performed. All efforts were made to minimize suffering and reduce the number of animals used.

Methods

Alcohol hangover model. Ethanol (EtOH), 15% w/v, was prepared by diluting a 95% stock solution of EtOH with 0.9% saline solution (SS). Animals received an injection (i.p.) of EtOH (3.8 g/kg BW) or saline (8:00 am). The EtOH dose applied in this work was previously used in other studies (Gilliam et al., 1990; Mollenauer et al., 1992; Brasser and Spear, 2002; Fee et al., 2004). According to our previous researches, AH onset was considered six hours after ethanol injection when blood alcohol concentration was close to zero (Bustamante et al., 2012).

Isolation of mouse cerebellum mitochondria. Six hours after injection, animals were killed by cervical dislocation in accordance with the directive systems of protection of vertebrate animals for scientific research. Control and AH animals were used in three different experiments. The cerebellum was rapidly removed and minced on ice, resuspended in MSH buffer (230 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4) supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600 g for 10 min at 4 °C. A protease inhibitor cocktail (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM PMSF and 1 μg/ml aprotinin) was added to the homogenates to discard nuclei and cell debris. The supernatant was decanted and centrifuged again at 8000 g for 10 min; the new mitochondrial pellet was washed twice in MSH without EDTA, in order to prevent calcium chelation. Mitochondria were stored on ice prior to the experiments. Protein was determined by the Lowry assay (Lowry et al., 1951). The isolated mitochondrial fraction corresponds to synaptic and non synaptic cerebellum mitochondria mainly from neurons and glial cells. Previous results from our laboratory have shown the level of cytosolic contamination of our mitochondrial preparations, by measuring the activity of the enzyme lactate dehydrogenase and the microsomal activity antimycin A-insensitive NADH-cytochrome *c* reductase in mitochondrial fractions, being less than 1.8% and 2.4% of the initial homogenate activity, respectively (Bustamante et al., 2000).

Mitochondrial respiratory function. Oxygen consumption by isolated cerebellum mitochondria was measured with a high resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria). Mitochondrial protein (0.5–1 mg/ml) was placed in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 6 mM malate plus glutamate or 7 mM succinate, 5 mM PO_4H_2K, 4 mM $MgCl_2$ (pH 7.4), and 0.1% free fatty acid bovine serum albumin, at 30 °C. State 3 was estimated by the addition of 0.5 mM ADP. Oxygen uptake was expressed in ng-atom O/min.mg protein and the respiratory control ratio (RCR) was calculated from the ratio of the state 3/state 4

respiratory rates with and without ADP, respectively (Estabrook, 1967). The mitochondrial fraction obtained from cerebellum tissue showed a RCR between 4.0–6.0.

Evaluation of mitochondrial respiratory complexes I–III, II–III and IV. NADH-cytochrome *c* reductase activity (complex I–III) was measured in cerebellum submitochondrial particles by following spectrophotometrically the reduction of cytochrome *c* at 550 nm (ε = 19.6 mM^{-1} cm^{-1}) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 0.1 mM cytochrome *c* and 0.5 mM KCN at 30 °C. Enzyme activity was expressed in nmol cytochrome *c* reduced per minute per mg of protein. Succinate cytochrome *c* reductase activity (complex II–III) was similarly determined and expressed, except that NADH was substituted by 20 mM succinate and 1 μM rotenone was added to prevent the possible reverse flux of electrons from complex II to I, yielding ROS formation. Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome *c* (Yonetani, 1967). The activity was expressed as k′/mg protein.

Mitochondrial transmembrane potential. Mitochondrial transmembrane potential was determined as follows: isolated mitochondria (25 μg/ml) were incubated at 37 °C for 20 min in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 4 mM $MgCl_2$ in the presence of 30 nM $DiOC_6$, a potentiometric probe that can be used for a direct measurement of transmembrane potential in cells and isolated mitochondria from different sources. The fluorescence changes were determined by cytometric measurements. Fresh mitochondria were prepared for each experiment and samples were protected from light until acquired by the cytometer. Auto-fluorescence of the mitochondrial preparation was measured as a probe loading control and 0.5 μM of the depolarizing agent FCCP was used as a positive control (Bustamante et al., 2011). $DiOC_6$ probe fluorescence was analyzed using the median value of the fluorescence events' distribution from each treatment. Histogram differences in $DiOC_6$ fluorescence were quantified in three independent experiments as the number of events which drop under the median value of the distribution using a common marker (M_1). M_1 was fixed on control median value representing approximately 50% of the fluorescent events (Karadayian et al., 2014). A higher number of events with low $DiOC_6$ fluorescence under M_1 reflect mitochondrial membrane depolarization. Quantification of results is shown as bar graph in which data are expressed as the percentage of control $DiOC_6$ fluorescence. The RCR was not affected by the concentration of the potentiometric probe used.

Analysis of mitochondrial permeability. Mitochondrial permeability and swelling occurrence were evaluated by two different methods: the analysis of side scatter (SSC) and the decrease in absorbance at 540 nm at 30 °C. For both determinations, cerebellum mitochondrial samples (0.5 mg/ml) from control and alcohol-treated animals were resuspended in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 2 mM $MgCl_2$, pH 7.4, at 30 °C.

Mitochondrial permeability was determined by analyzing light-scattering properties by flow cytometry (Mattiasson et al., 2003; Lecoeur et al., 2004). The differences in SSC were evaluated and analyzed as the induction of mitochondrial membrane permeability. This parameter allows analyzing the differences in membrane granulocity and cellular complexity. In order to evaluate the induction of mitochondrial transition pore, each mitochondrial sample was exposed to 200 μM Ca^{2+}. Previous research showed that 200 μM Ca^{2+} is able to induce MPT (mitochondrial permeability transition pore) evidenced by a clear decrease in SSC in mouse brain cortex mitochondria (Bustamante and Lores-Arnaiz, 2010). As positive and negative controls, mitochondria samples of each treatment were exposed during 5 min to 10 μg/ml alamethicin or 2 μM cyclosporin A (CsA) respectively. In this sense, while the ionophore alamethicin induces unspecific maximal mitochondrial swelling (Hansson et al., 2003), CsA induces inhibition of MPT obtaining similar SSC values as untreated mitochondria (Friberg et al., 1999; Domañska-Janik et al., 2004). Differences in the observed SSC for mitochondrial samples were quantified in three independent experiments as the number of events which drop under the median value of the histogram distribution using a common marker (M1) (Bustamante et al., 2014). Results were included in a bar graph which shows the results expressed as the percentage of control SSC. A lower value of SSC would reflect an increase of mitochondrial permeability.

In addition, cerebellum mitochondrial swelling was monitored by the decrease in absorbance at 540 nm ($\delta A_{540\ nm}$/min mg protein) after addition of 200 μM Ca^{2+} at 30 °C. Swelling was obtained by drawing a tangent to the plot of absorbance/time at its steepest point (Bustamante et al., 2011).

Mitochondrial superoxide production. Mitochondrial superoxide relative production was determined as follows: isolated mitochondria (25 μg/ml) were incubated at 37 °C for 20 min in MSH buffer supplemented with 5 mM succinate, 5 mM glutamate, 1 mM phosphate and 4 mM $MgCl_2$ in the presence of 2.5 μM MitoSOX, a potentiometric probe that can be used for direct measurement of mitochondrial superoxide by flow cytometry. Fresh mitochondria were prepared for each experiment and samples were protected from light until acquired by the cytometer FAC-SCAN Ortho-Cytoron (Bustamante et al., 2004). Auto-fluorescence was evaluated in samples without the probe. In addition, 1 μM antimycin, an ubiquinol cytochrome *c* reductase inhibitor, was added as a positive control. MitoSOX fluorescence was analyzed using the median value of the fluorescence events distribution from each treatment. Histogram differences in MitoSOX fluorescence were quantified in three independent experiments as the number of events which drop under the median value of the distribution using a common marker (M_1). M_1 marker was fixed on control median value representing approximately 50% of the

fluorescent events. A lower number of events with low MitoSOX fluorescence under M_1 would reflect an increased superoxide production. Quantification of results is shown as bar graph in which data are expressed as the percentage of control MitoSOX fluorescence.

Mitochondrial hydrogen peroxide production. Hydrogen peroxide generation was determined in intact cerebellum mitochondria by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365–450 nm as λexc − λem at 37 °C (Boveris, 1984). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin and 0.3 μM SOD to ensure that all superoxide ($O_2^{\cdot-}$) was converted to H_2O_2; 6 mM succinate plus 6 mM glutamate or 6 mM malate plus 6 mM glutamate were used as substrates.

To determine monoamine oxidase (MAO) involvement in H_2O_2 production, mitochondrial samples were pre-incubated with a MAO inhibitor (10 μM deprenyl) during 2 min. In addition, 5 μM rotenone was used to inhibit complex I enzymatic activity. Calibration was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min.mg protein. Hydrogen peroxide production was highly sensitive to catalase addition (3.500 U/ml).

MAO activity. MAO activity was measured in cerebellum submitochondrial membranes from control and hangover mice by following spectrophotometrically the oxidation of kynuramine at 30 °C, in a reaction medium containing 50 mM phosphate buffer (pH 7.4). Kinetics were followed at 360 nm (ε = 4.28 mM^{-1} cm^{-1}) (Weissbach et al., 1960).

Antioxidant enzyme activities. Glutathione peroxidase, catalase and SOD activities were determined in cerebellum cytosolic and mitochondrial fractions by spectrophotometric measurements. Glutathione peroxidase activity was measured following NADPH oxidation at 340 nm in the presence of 0.17 mM reduced glutathione, 0.2 U/ml glutathione reductase and 0.5 mM tert-butyl hydroperoxide and expressed in mU/mg protein (Flohé and Günzler, 1984). Catalase activity was assayed following absorbance decrease of hydrogen peroxide at 240 nm, in a medium containing 50 mM phosphate buffer and 10 mM H_20_2 (pH 7.2), and expressed in pmoles enzyme/mg protein (Chance et al., 1979). SOD activity was determined by measuring the inhibition of autocatalytic adrenochrome formation rate in a reaction medium containing 1 mM epinephrine and 50 mM glycine-NaOH (pH 10.2) and expressed in U/mg protein (Misra and Fridovich, 1972).

Nitric oxide synthase expression by Western blot. Submitochondrial membranes (80 μg) were separated by SDS–PAGE (7.5%), blotted onto a nitrocellulose membrane (Bio-Rad, München, Germany) and probed primarily with rabbit polyclonal antibodies (dilution 1:500) for the nNOS, epitope corresponding to amino acids 2–300, mapping to the amino terminus of NOS I. Then, the nitrocellulose membrane was incubated with a secondary goat anti-rabbit antibody conjugated with HRP (dilution 1:5000), followed by development of chemiluminescence with the ECL reagent (Santa Cruz Biotechnology) for 2–4 min (Lores-Arnaiz et al., 2007). VDAC was used as loading control. Densitometric analysis of nNOS and VDAC bands was evaluated through NIH Image J 1.47b software and expressed as the ratio of nNOS/VDAC taking control ratio as 100%. All experiments were performed in triplicate.

Nitric oxide production associated with the mitochondria. NO production by nNOS was measured in cerebellum submitochondrial membranes (0.2–0.5 mg/ml) using a double-beam dual-wavelength spectrophotometer, following the oxidation of oxyhemoglobin (25 μM in heme) to methemoglobin at 577–591 nm ($\varepsilon_{577\text{-}591}$ = 11.2 mM^{-1} cm^{-1}), sensitive to L-NNA inhibition (Boveris et al., 2002; Bustamante et al., 2008) at 37 °C. The reaction medium contained 50 mM phosphate buffer (pH 5.8), 50 μM L-arginine, 100 μM NADPH, 10 μM dithiothreitol, 1 mM $CaCl_2$, 0.5–1.0 mg submitochondrial protein/ml and 25 μM oxyhemoglobin (expressed per heme group). In order to avoid the presence of $O_2^{\cdot-}$ and H_2O_2, Cu–Zn SOD and catalase at 4 and 0.1 μM respectively, were also added to the reaction medium (Lores-Arnaiz et al., 1999). The results were expressed as nmol of NO per minute per milligram protein. NO production associated with the mitochondria, is also strongly dependent on the availability of arginine; this compound is present in the central nervous system mitochondria in non-limiting concentrations (Lores-Arnaiz et al., 2004).

Statistical analysis

Results are presented as mean ± SEM. Prior to each analysis, test variables were checked for normality; all data were evaluated by the Kolmogorov–Smirnov test in order to follow a posterior parametric or nonparametric statistical analysis. The analysis of the results was performed using unpaired Student t-test or ANOVA and post hoc Tukey in order to analyze the significance of differences between groups. IBM SPSS Statistics (22.0 version) software was used and a difference was considered to be statistically significant when $p < 0.05$.

RESULTS

Cerebellum mitochondrial respiratory function

Malate-glutamate or succinate-dependent oxygen consumption was measured in state 4 (at rest or controlled respiration) and in state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis). The RCR (the most sensitive indicator of mitochondrial oxidative phosphorylation coupling) was calculated as the relationship between state 3 and state 4 respiration rates. Table 1 shows oxygen consumption rates of intact cerebellum mitochondria isolated from control and AH. Cerebellum control mitochondria showed a respiratory control of 6.2–6.9 indicating adequate integrity and mitochondrial function. At the onset of AH, state 4 oxygen uptake was 2.3 and 1.9-fold increased with malate-glutamate ($p < 0.05$) and succinate

Table 1. Effect of alcohol hangover on cerebellum mitochondrial oxygen consumption

Substrate/Condition	O_2 consumption (ng-atom O/min mg protein)		RCR
	State 4	State 3	
Mal-Glu			
Control	4.2 ± 0.8	25.3 ± 4.1	6.2 ± 0.6
Alcohol hangover	9.5 ± 0.6*	26.8 ± 1.1	2.8 ± 0.1*
Succinate			
Control	3.3 ± 0.4	21.9 ± 0.7	6.9 ± 0.5
Alcohol hangover	6.4 ± 0.3**	23.1 ± 0.9	3.6 ± 0.7*

Malate-glutamate and succinate were used as substrates as described in Materials and Methods. Values represent mean ± SEM of 4–6 individual mitochondria samples, each obtained from a pool of cerebellum from five mice.
ANOVA Tukey test: $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with control group.

($p < 0.01$) leading to a reduction in respiratory control of 55% and 48% respectively as compared with control animals ($p < 0.05$). Levels of state 3 respiratory rates were not significantly different from controls.

Mitochondrial respiratory complexes I-III, II-III and IV

Respiratory complex activities were measured in cerebellum submitochondrial particles from control and hangover groups. Results are shown in Table 2. Decreases of 38% ($p < 0.001$) and 16% ($p < 0.05$) were found in NADH-cytochrome *c* reductase (Complex I-III) and cytochrome oxidase (Complex IV) respectively. Succinate-cytochrome *c* reductase (Complex II–III) activity in cerebellum submitochondrial membranes was not affected by AH.

Mitochondrial transmembrane potential

The effect of AH on mitochondrial transmembrane potential was evaluated in cerebellum by flow cytometry after loading mitochondria with the potentiometric probe $DiOC_6$. Dotblot of FSC-H vs. SSC-H indicating the gated mitochondrial population is shown in Fig. 1a. Results show a decrease in $DiOC_6$ fluorescence median in the ethanol hangover group (6.21) compared with control group (10.27) (Fig. 1b, c). Also, a significant decrease in $DiOC_6$ fluorescence was observed after treatment with the depolarizing agent FCCP (Median: 3.08) (Fig. 1d). Qualitative analysis shows the effect of AH and FCCP on probe fluorescence events in comparison with control group (Fig. 1e). Quantification results show a 53% decrease in mitochondrial membrane potential in the AH group ($p < 0.01$; Fig. 1f). As expected, the positive control with FCCP treatment showed a 75% decrease in mitochondrial membrane potential ($p < 0.001$; Fig. 1f).

Table 2. Effect of alcohol hangover on enzymatic activity of cerebellum mitochondrial respiratory complexes

Experimental group	Enzymatic activity		
	Complex I–III (nmol/min mg prot)	Complex II–III (nmol/min mg prot)	Complex IV (k′/mg prot)
Control	183 ± 4	41 ± 2	184 ± 4
Alcohol hangover	113 ± 4***	45 ± 3	155 ± 4*

Values represent the mean ± SEM of 4–6 individual mitochondria samples, each obtained from a pool of cerebellum from five mice.
ANOVA Tukey test: $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with control group.

Analysis of mitochondrial permeability

Mitochondrial permeability and swelling occurrence were evaluated by two different methods: the analysis of SSC and the decrease in absorbance at 540 nm at 30 °C. Histograms showing SSC for the different experimental conditions are shown in Fig. 2(a–f). The analysis shows a clear decrease in SSC median in hangover mitochondria (170.4) compared with control samples (191.1). Also, SSC median value of Ca^{2+}-loaded control mitochondria (175.2) was significantly lower than unloaded samples (191.1). Calcium addition to hangover mitochondria resulted in a decrease in SSC (176.24) which was not significantly different from either hangover or Ca^{2+}-loaded control conditions. Quantification analysis showed a 30% decrease in SSC both after Ca^{2+} or alamethicin treatment compared with control group ($p < 0.001$; Fig. 2h). AH showed a 20% decrease in SCC compared with control group, which was not further modified by the addition of Ca^{2+} ($p < 0.05$; Fig. 2h). As expected, the negative control with CsA induced inhibition of MPT obtaining similar SSC values as untreated mitochondria.

The analysis of the decay in absorbance at 540 nm showed that AH induced a significant increase in MPT compared with control ($p < 0.05$, Fig. 3). As expected, MPT was significantly increased by Ca^{2+} addition in control mitochondria ($p < 0.05$, Fig. 3). MPT induction after Ca^{2+} addition to hangover samples did not differ from MTP values for hangover mitochondria.

Mitochondrial superoxide production

Mitochondrial superoxide relative production was determined by flow cytometry using the potentiometric probe MitoSOX. Dotblot of FSC-H vs. SSC-H indicating the gated mitochondrial population is shown in Fig. 4a. AH condition induced an increase in MitoSOX fluorescence median compared with control group (15.6 vs. 11.09, respectively; Fig. 4b, c). As expected, antimycin provoked a clear increase in the fluorescence median compared with control group (26.9; Fig. 4d). The quantification of fluorescence events from three independent experiments indicates a 25% increase in MitoSOX fluorescence in the hangover condition compared with controls ($p < 0.05$; Fig. 4e). Antimycin induced a 75% increase in MitoSOX fluorescence ($p < 0.01$; Fig. 4e). Thus, AH produced a significant increment in superoxide production in cerebellum mitochondria.

Mitochondrial hydrogen peroxide production

Hydrogen peroxide production rates were determined using succinate-glutamate or malate-glutamate as

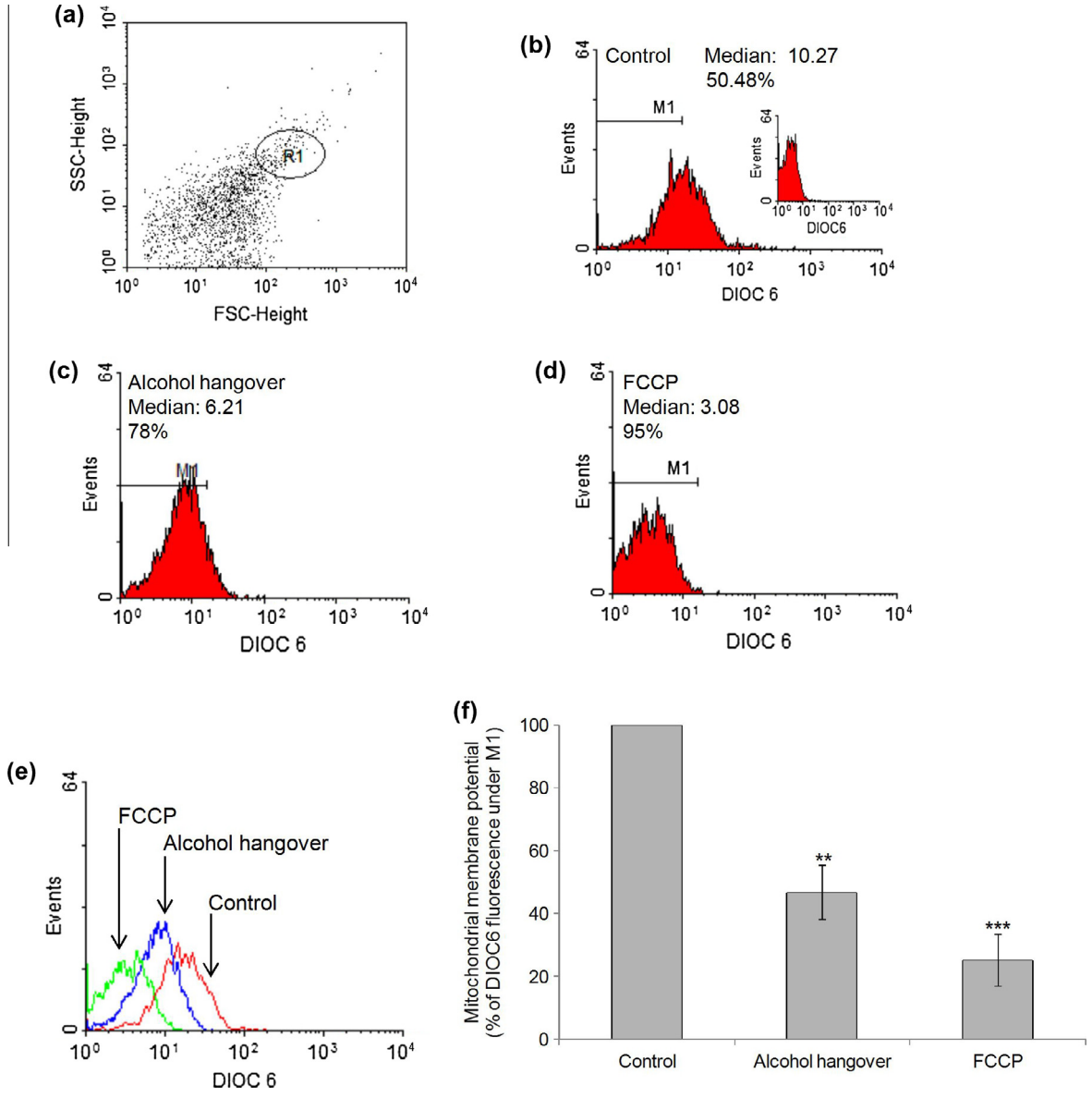

Fig. 1. Mitochondrial membrane potential ($\Delta\psi m$) through the changes in $DiOC_6$ fluorescence intensity. (a): Dotblot of FSC-H vs. SSC-H indicating a gated mitochondrial population (R1). (b–d): Histograms of gated mitochondrial events (R1) vs. relative fluorescence intensity (FL-1), corresponding to three experimental conditions: (b): control, (c): alcohol hangover and (d): FCCP-treated mitochondria. Samples without probe were used for autofluorescence (insets). (e): Overlapped histograms showing the effect of the three experimental conditions on $\Delta\psi m$. (f): Bar scheme of $DiOC_6$ fluorescence quantification representing mean ± SEM of three different experiments. Fluorescence events were quantified as the number of events which drop under a common marker M1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. ANOVA, Tukey's test ($^{**}p < 0.01$, $^{***}p < 0.001$). Each histogram represents a typical experiment which was performed in triplicate.

substrates in the presence of inhibitors of MAO (deprenyl) and of complex I (rotenone) in intact cerebellum mitochondria isolated from the two studied groups. The use of succinate-glutamate to determinate ROS production was based on the fact that these substrates induce the highest production of H_2O_2 (Muller et al., 2008). Results are shown in Fig. 5. Control H_2O_2 production rates were 90% and 70% increased in the hangover condition when succinate-glutamate and malate-glutamate were used as substrates respectively ($p < 0.05$). Deprenyl induced a 32% decrease in H_2O_2 production in control mitochondria ($p < 0.05$) while a 44% decrease was found in the hangover condition compared with the same group without the inhibitor ($p < 0.05$). The combined addition of rotenone and deprenyl induced 88% and 94% decreases in H_2O_2 production in the hangover group compared with the hangover sample in the presence of deprenyl alone or without any inhibitor respectively ($p < 0.05$).

MAO activity

Monoamine oxidase activity was determined in mitochondrial fractions from control and ethanol hangover mice. MAO activity was 79% increased in the AH group compared with control ($p < 0.001$, Table 3).

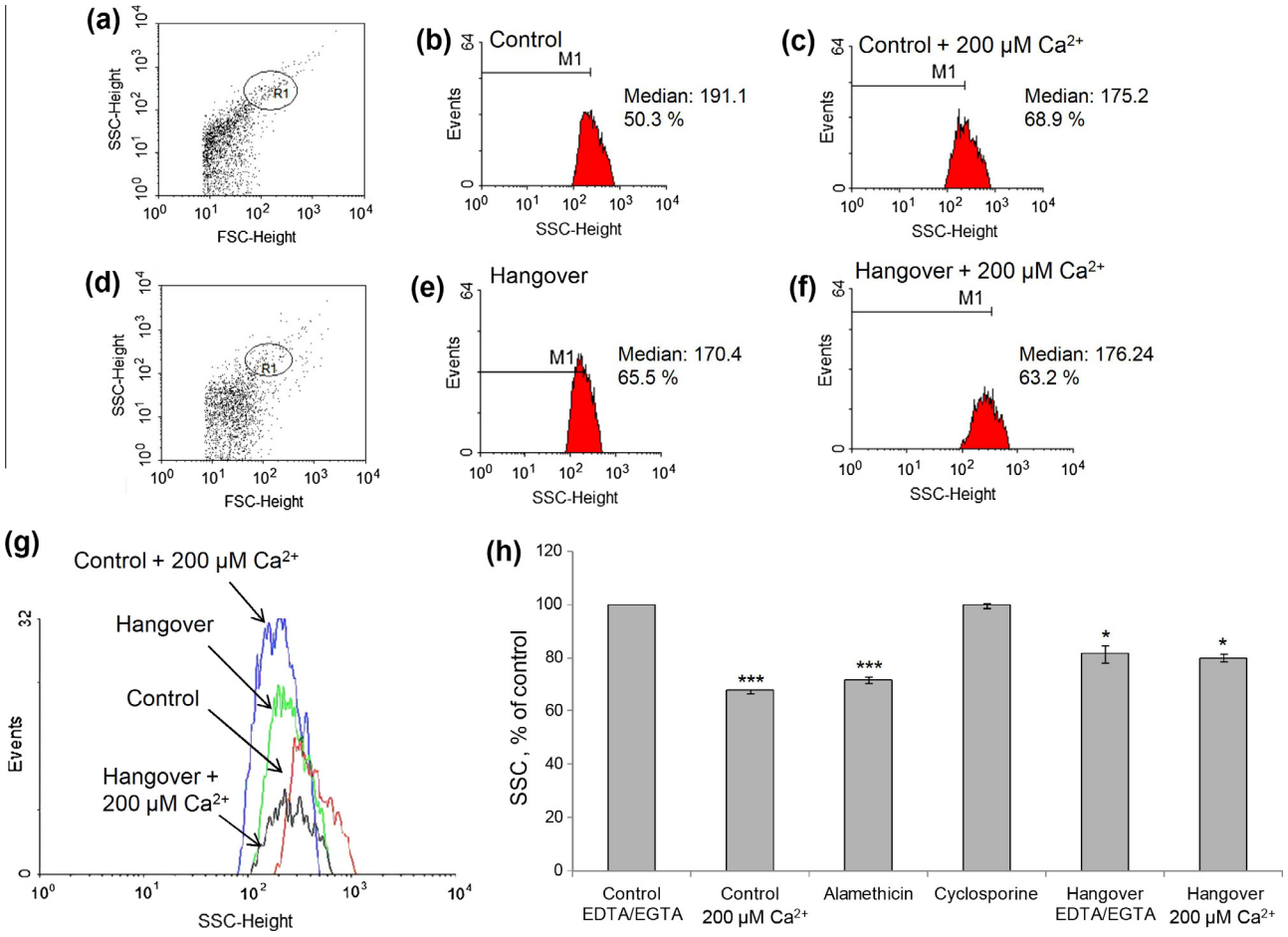

Fig. 2. Evaluation of mitochondrial permeability by flow cytometry measurements of SSC. (a, d): Dotblot of FSC-H vs. SSC-H indicating a gated mitochondrial population (R1) for control (a) and hangover (d) conditions. (b–d): Histograms of gated mitochondrial events (R1) vs. SSC, corresponding to three experimental conditions: (b): control, (c): control treated with 200 µM, (e): alcohol hangover and (f): hangover treated with 200 µM Ca^{2+}. (g): Overlapped histograms showing SSC of purified cerebellum mitochondria samples from control, control treated with 200 µM Ca^{2+}, alcohol hangover and hangover treated with 200 µM Ca^{2+}. Each histogram represents a typical experiment which was performed in triplicate. (h): Bar scheme represents mean ± SEM of the number of events which drop under a common marker M1 (fixed at the median value of the control histogram), taking control SSC as 100%. A lower value of SSC would reflect a loss of mitochondrial complexity or granulosity. ANOVA, Tukey's test ($^{*}p < 0.05$, $^{***}p < 0.001$; compared with control). Alamethicin and cyclosporine A were used as positive and negative controls, respectively.

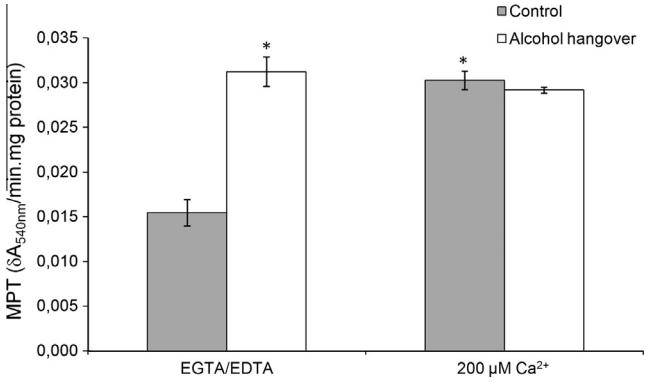

Fig. 3. Evaluation of mitochondrial permeability by spectrophotometry. Cerebellum mitochondrial swelling was monitored by the decrease in absorbance at 540 nm ($\delta A_{540\ nm}$/min mg protein) after addition of 200 µM Ca^{2+} at 30 °C. Bars represent mean ± SEM of three different swelling experiments. ANOVA, Tukey's test: $^{*}p < 0.05$, significantly different from control with EGTA/EDTA. Bar colors indicate: gray, control; white, alcohol hangover.

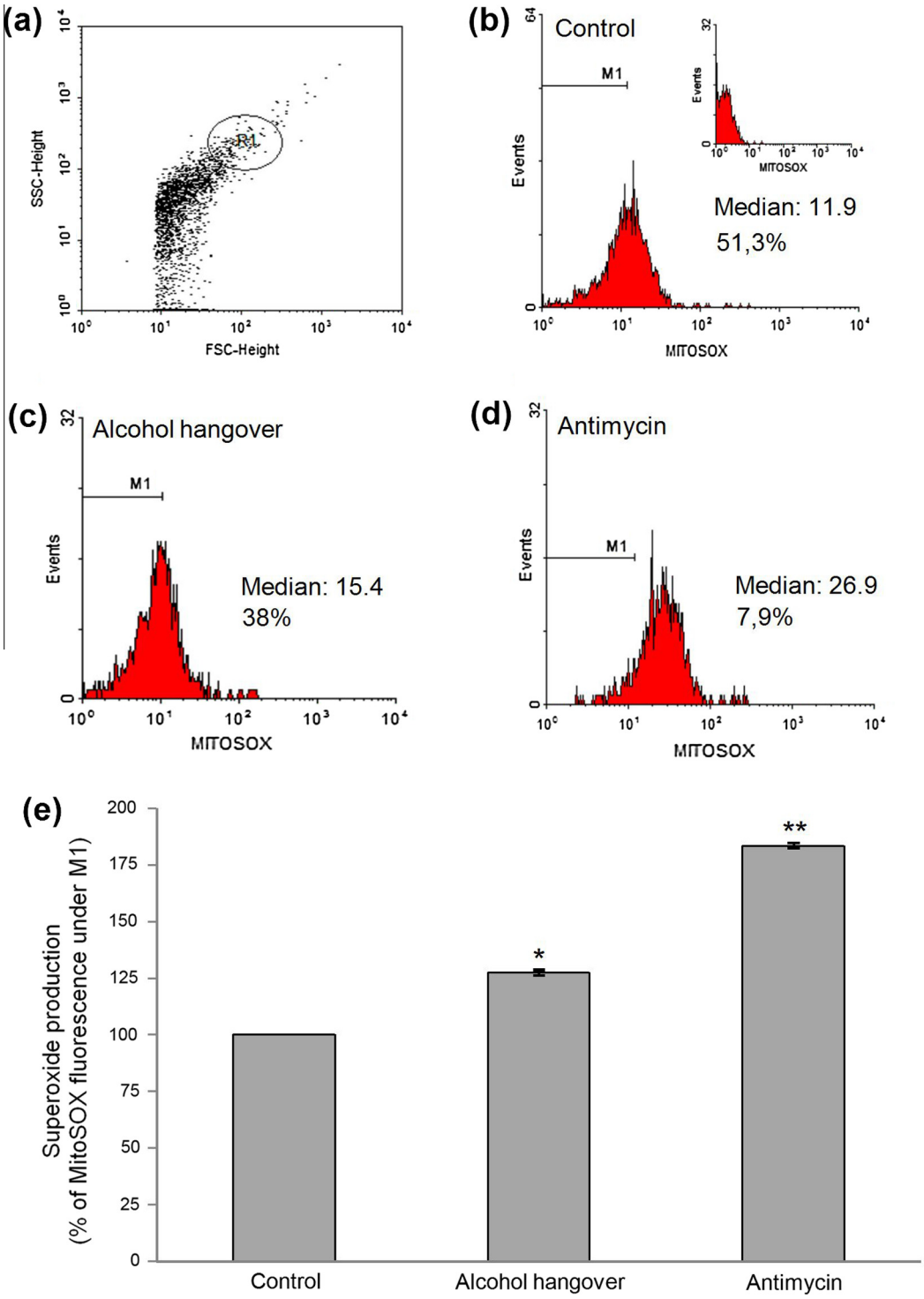

Fig. 4. Mitochondrial superoxide anion level. Cerebellum mitochondria fractions were loaded with the potentiometric probe MitoSOX and direct measurements of mitochondrial superoxide were obtained by flow cytometry. (a): Dotblot of forward scatter (FSC) vs. SSC indicating the gated mitochondrial population (R1). Samples without probe were used for autofluorescence (insets), (b–d): Fluorescence histograms (FL-1) corresponding to three experimental conditions: (b): control, (c): alcohol hangover and (d): antimycin as a positive control, (e): Bar scheme of MitoSOX fluorescence quantification representing mean ± SEM of three different experiments. Fluorescence events were quantified as the number of events which drop under a common marker M1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. ANOVA, Tukey's test ($^{*}p < 0.05$, $^{**}p < 0.01$). Each histogram represents a typical experiment which was performed in triplicate.

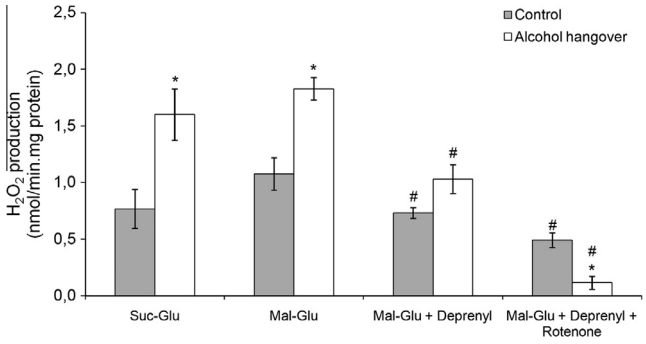

Fig. 5. Mitochondrial hydrogen peroxide production rate. Mitochondria were supplemented with 6 mM succinate plus 6 mM glutamate or 6 mM malate plus 6 mM glutamate, as described under Materials and methods. Deprenyl and rotenone were used as inhibitors of MAO and complex I, respectively. Bars represent mean ± SEM of four-to-six individual mitochondria samples, each obtained from a pool of 5 mice cerebellum. ANOVA, Tukey's test: $^{*}p < 0.05$, compared with control condition; $^{\#}p < 0.05$, compared with same condition without the inhibitors. Bar colors indicate: gray, control; white, alcohol hangover.

Antioxidant enzyme activities

Glutathione peroxidase, catalase and SOD activities were determined in cytosol and mitochondrial fractions from cerebellum by spectrophotometric measurements (Table 3). Results indicate that AH induced a significant decrease in catalase and glutathione peroxidase activities. Particularly, catalase activity was 42% and 15% decreased in cytosolic and mitochondrial fractions respectively ($p < 0.01$) and glutathione peroxidase activity was 32% and 57% decreased in cytosolic and mitochondrial fractions respectively ($p < 0.01$). Together with this, SOD-1 and SOD-2 were two and fourfold increased in the hangover group, as compared with controls ($p < 0.05$) (see Table 3).

NO production associated with the mitochondria

Cerebellum NO production associated with the mitochondria was studied by evaluating nNOS expression and NO production at the onset of AH. Brain NOS associated with mitochondrial membranes has been previously identified by Western blot analysis mainly as nNOS and also but to a lesser extent as endothelial nitric oxide synthase (eNOS) and iNOS (Lores-Arnaiz et al., 2004). In this study, Western blot analysis for nNOS in association with cerebellum submitochondrial membranes is shown in Fig. 6a-b. Results showed that nNOS protein expression was 52% decreased by the hangover condition compared with control group ($p < 0.001$). No significant differences were found in cerebellum NO production at the onset of AH, being NO production values 0.979 ± 0.04 and 0.943 ± 0.08 nmoles/min mg for control and hangover conditions, respectively.

DISCUSSION

Previous observations by our laboratory have demonstrated mitochondrial dysfunction and free radical production at the onset of AH in the brain cortex 6 h after acute ethanol exposure in association with a reduction in motor performance (Bustamante et al., 2012). In the present study, we found that also in other brain areas such as cerebellum, mitochondrial respiratory function was impaired by the hangover condition. Particularly, respiratory control was significantly reduced by an increase in state 4 both with malate-glutamate and succinate as substrates. This effect was different from what we previously reported for the brain cortex in which mitochondrial respiratory control was reduced by a decreased malate-glutamate-dependent state 3 respiratory rate (Bustamante et al., 2012). The activity of enzymatic complexes of the mitochondrial respiratory chain was also modified in cerebellum by the hangover condition. In fact, the activities of Complexes I–III and IV were clearly reduced while Complex II-III activity was conserved. The decrease in Complex IV activity by the hangover condition was not enough to affect state 3 respiratory rate. In fact, according to Davey et al. (1997) complex IV

Table 3. Effect of alcohol hangover on the activity of antioxidant enzymes and monoamine oxidase in subcellular fractions

Enzyme	Subcellular fraction	Control	Alcohol hangover
SOD (U/mg protein)	Cytosol	8.4 ± 1.2	35.9 ± 1.1*
	Mitochondria	13.7 ± 3.4	28.1 ± 7.1*
CAT (pmol/mg protein)	Cytosol	0.123 ± 0.004	0.071 ± 0.002**
	Mitochondria	0.114 ± 0.001	0.097 ± 0.002**
GPx (mU/mg protein)	Cytosol	229.4 ± 4.7	156.8 ± 2.9**
	Mitochondria	104.6 ± 12.5	45.1 ± 10.6**
MAO (nmol/min mg protein)	Mitochondria	14.4 ± 1.1	25.7 ± 2.2***

Results are expressed as mean values ± SEM of 4–6 individual mitochondria samples, each obtained from a pool of cerebellum from five mice. ANOVA Tukey test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with control group.

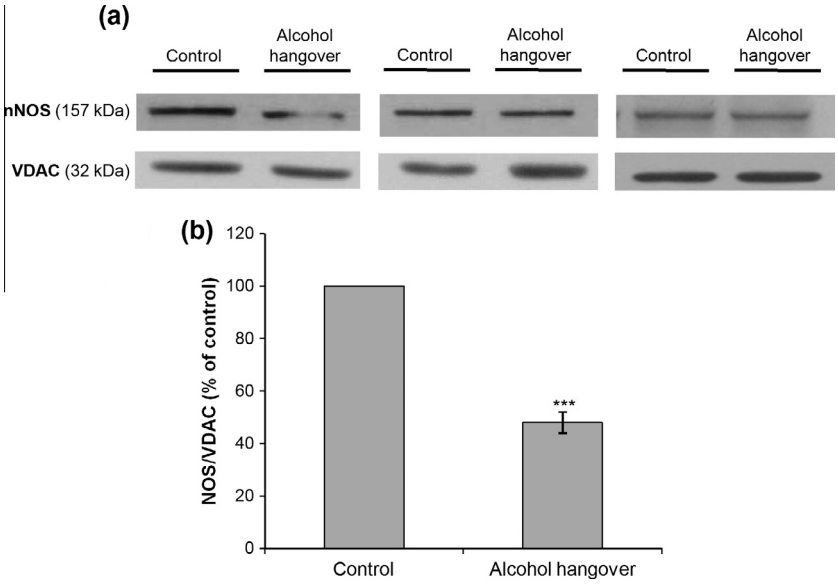

Fig. 6. Mitochondrial nitric oxide synthase expression and activity. (a): Western blot analysis of nNOS protein expression in cerebellum mitochondria. VDAC was used as loading control. Results are representative of three independent studies. (b): Bars represent nNOS/VDAC ratio obtained after densitometric analysis. Data (mean ± SEM) are expressed as % of the control group (set at 100%). Student *t*-test. (***$p < 0.001$).

activity should be 50–60% reduced before respiration rates and ATP synthesis could be severely compromised.

Our results regarding the electron transport chain activity led us to hypothesize that being complex II–III activity preserved in the hangover condition, the electron flux may be redirected to complex I by reverse electron flow leading to an increased state 4 oxygen consumption, with the consequent increase in electron leakage. In accordance, mitochondria became less efficient leading to a decrease in respiratory control.

It was previously established that changes in mitochondrial respiratory complexes could induce the opening of MPT pore which is one of the molecular mechanisms causing mitochondrial dysfunction (Petronilli et al., 1994), and is also closely associated with alteration of cellular Ca^{2+} homeostasis, impairment of the electron transport chain, increased oxidative stress, mitochondrial swelling and loss of transmembrane potential (Kim et al., 2003b). In our study, the effect of AH in cerebellum oxygen uptake and complex activities was also accompanied by changes in mitochondrial membrane permeability, as evidenced by changes in light-SSC by flow cytometry and the decay of absorbance at 540 nm in the mitochondrial population. As a whole, the results showed that AH induced an increase in mitochondrial permeability, indicating the presence of swelling. Also, a significant mitochondrial depolarization was observed in the cerebellum after acute ethanol exposure. Surprisingly, calcium added to hangover samples did not exacerbate the swelling due to alcohol residual effect. In line with this, King and colleagues (2014) evidenced that the addition of calcium to liver mitochondria from mice chronically exposed to ethanol exhibited a different profile in the swelling onset and intensity depending on calcium concentration. Specifically, even when mitochondria from ethanol fed mice treated with 8-nmol calcium exhibited an earlier MPT onset than controls, the addition of higher calcium concentrations (e.g. 40 nmol) did not modify swelling onset.

Interesting to note is that our data show that AH decreases $\Delta\psi m$ together with an increase in state 4 respiration. It has been suggested that mitochondrial uncoupling protein expression may be involved in the regulation of the complex responses to ethanol in the central nervous system (Horvath et al., 2002). However, even when a mild uncoupling should be part of the initial mitochondrial response, our results present evidences of swelling and strong mitochondrial dysfunction by AH. In fact, during hangover, mitochondria showed a strong depolarization, changes in permeability and inhibition of respiratory complexes I and IV. Moreover, as previously stated, complex II-III activity was preserved in the hangover condition and the electron flux could be redirected to complex I by reverse electron flow, increasing state 4 respiration.

Our present results indicate that AH induced a significant increase in superoxide and hydrogen peroxide production in cerebellum mitochondria, similarly to our previous observations in the brain cortex (Bustamante et al., 2012). In accordance, Heaton et al. (2002) evidenced an increment of active oxygen species production in postnatal rat's cerebellum after acute ethanol administration. Also it has been suggested that the increase of free radical production in the cerebellum could result in a permanent loss of GABAergic Purkinje cells (Ramezani et al., 2012). Together with this, Hoek et al. (2002) established that excessive free radical production due to acute EtOH consumption could be involved in mitochondrial membrane permeability loss associating this to an increase in proapoptotic signals.

In the present study, the source of H_2O_2 generation after ethanol hangover was investigated using different inhibitors. The results showed that the huge increment in H_2O_2 production could be due not only to complex I but also to MAO activity since the inhibition of this

enzyme with deprenyl induced a decrease in H_2O_2 production and this effect became stronger when complex I was also inhibited. The contribution of MAO activity to the overall production of H_2O_2 was also evidenced by a significant increase in its enzyme activity.

In order to analyze the role of antioxidant enzymes in the alterations in the redox state associated with the hangover condition, we studied the activity of antioxidant enzymes. The results showed that both catalase and glutathione peroxidase activities were decreased and, in parallel, Cu–Zn SOD and Mn-SOD activities were significantly increased by the hangover. These facts strongly support the idea that the increase in H_2O_2 levels is due in part to its decreased catabolism by the low catalase and glutathione peroxidase activities. Furthermore, the increased activity in both SOD isoforms could also contribute to the higher increase in H_2O_2 concentration during hangover as compared with the superoxide increments.

Related to this concept, Assunção et al. (2008) has postulated that one of the reasons by which the cerebellum is one of the brain areas most affected by EtOH intake would be the low basal level of antioxidant defenses and the presence of a large quantity of oxidizable lipids.

Previous researches indicate that alcohol dependence does not only alter oxidative status, but also induces nitrosative species imbalance in the rat brain altering mitochondrial membrane properties (Reddy et al., 2013). In this sense, we evaluated cerebellum NO metabolism at the onset of AH. Our results indicate a clear decrease in NOS expression without changes in NO production between alcohol and control mice, indicating that NO metabolism is partially impaired by the hangover condition. In line with this, Zima et al. (2001) established that chronic ethanol exposure decreases nNOS activity in rat cerebellum fibers explaining the motor disturbances observed during ethanol withdrawal. The difference between these findings and ours regarding nNOS activity response to EtOH could be explained considering the type of ethanol exposure; while acute EtOH administration could decrease the enzyme expression, chronic EtOH administration decreases also its activity. The decreased NOS expression could impair vital physiological processes like vasodilatation and neurotransmission (Thippeswamy et al., 2006). In accordance to this fact, previous works from Lores-Arnaiz et al. (2006, 2007, 2010) showed that the modulation of NO levels by enriched environments could improve cognition performance suggesting a role of NO in learning and memory processes. Accordingly, Singhal et al. (1999) suggested that the inhibitory effects on NOS expression and activity by acute or chronic EtOH exposure could trigger apoptotic process in neutrophil cells. Related to this, it was recently established that the inhibitory effects of EtOH on the cerebellum could be due to GABAergic signals such as the suppression of GABA post-synaptic activity receptors or the increment of GABA vesicles release by nNOS inhibition (Kaplan et al., 2013).

In summary, the present work demonstrates that the physiopathological state of AH involves mitochondrial dysfunction in the mouse cerebellum. The impairment in mitochondrial function in the central nervous system together with cell redox imbalance could play an important role in neuronal damage. Cerebellar vulnerability to oxidative damage was proven in rats during ethanol-withdrawal leading to movement disorders (Jung, 2014). Finally, being the cerebellum an integrative area of important sensory and motor pathways, the present results would contribute to the understanding of the mechanisms involved in the physiological disturbances associated with binge drinking.

CONCLUSIONS

This study shows that AH induces mitochondrial dysfunction in the mouse cerebellum six hours after of acute ethanol exposure. Impairment in mitochondrial energetic metabolism includes deprivation in oxygen uptake, inhibition in respiratory complexes, changes in mitochondrial membrane permeability, decrease in transmembrane potential, increase in superoxide anion and hydrogen peroxide production and impairment in NO metabolism.

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