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EFFECT OF MELATONIN ON MOTOR PERFORMANCE AND BRAIN CORTEX MITOCHONDRIAL FUNCTION DURING ETHANOL HANGOVER

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Abstract—Increased reactive oxygen species generation and mitochondrial dysfunction occur during ethanol hangover. The aim of this work was to study the effect of melatonin pretreatment on motor performance and mitochondrial function during ethanol hangover. Male mice received melatonin solution or its vehicle in drinking water during 7 days and i.p. injection with EtOH (3.8 g/kg BW) or saline at the eighth day. Motor performance and mitochondrial function were evaluated at the onset of hangover (6 h after injection). Melatonin improved motor coordination in ethanol hangover mice. Malate–glutamate-dependent oxygen uptake was decreased by ethanol hangover treatment and partially prevented by melatonin pretreatment. Melatonin alone induced a decrease of 30% in state 4 succinate-dependent respiratory rate. Also, the activity of the respiratory complexes was decreased in melatonin-pretreated ethanol hangover group. Melatonin pretreatment before the hangover prevented mitochondrial membrane potential collapse and induced a 79% decrement of hydrogen peroxide production as compared with ethanol hangover group. Ethanol hangover induced a 25% decrease in NO production. Melatonin alone and as a pretreatment before ethanol hangover significantly increased NO production by nNOS and iNOS as compared with control groups. No differences were observed in nNOS protein expression, while iNOS expression was increased in the melatonin group. Increased NO production by melatonin could be involved in the decrease of succinate-dependent oxygen consumption and the inhibition of complex IV observed in our study. Melatonin seems to act as an antioxidant agent in the ethanol hangover condition but also exhibited some dual effects related to NO metabolism.
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Key words: melatonin, ethanol hangover, motor coordination, mitochondrial function, mitochondrial membrane potential, oxidative stress.

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) is a highly conserved hormone mostly secreted by the pineal gland during the night in all known mammals including humans (Cardinali and Pevet, 1998).

Melatonin and its metabolites have been proven to be endogenous free-radical scavengers and broad-spectrum antioxidants (Tan et al., 2000; Manda et al., 2007; Reiter et al., 2008). Moreover, melatonin prevents neuronal cell death in a large number of models of brain damage where oxidative stress is involved, protecting for instance against seizures induced by kainate, glutamate, and *N*-methyl-D-aspartate (NMDA) (Lapin et al., 1998) and acting as a neuroprotector and an antioxidant agent both *in vivo* and *in vitro* (Cagnoli et al., 1995; Giusti et al., 1996). Moreover, effects of melatonin on rodent behavior have also been demonstrated; it induces sedation, elevates the threshold of pain perception, exhibits anxiolytic effects and directly resets circadian rhythms (Golombek et al., 1996).

Because of its small size and amphiphilic nature, melatonin easily reaches all cellular and subcellular compartments including mitochondria (Menendez-Pelaez and Reiter, 1993). Most of the beneficial consequences resulting from melatonin administration may depend on its effect on mitochondrial physiology (Jou et al., 2005, 2007; Peng et al., 2006). Melatonin's functions as an antioxidant include direct free radical scavenging, stimulation of antioxidant enzymes, increment of mitochondrial efficiency in relation to oxidative phosphorylation, enhancement of the electron transport chain activity and exacerbation of the effect of other antioxidants (Carpentieri et al., 2012).

Reactive oxygen species are generated during ethanol metabolism, causing oxidative stress and lipid peroxidation in brain (Calabrese et al., 1998; Comporti et al., 2010).

Ethanol hangover begins when alcohol disappears from blood and is characterized by unpleasant physical and psychological symptoms in humans (Kim et al.,

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Abbreviations: BAC, blood alcohol concentration; $DiOC_6$, 3,3″-dihexyloxacarbocyanine iodide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; H_2O_2, Hydrogen peroxide; HEPES, hydroxyethyl piperazineethanesulfonic acid; HRP, horseradish peroxidase; iNOS, Inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; L-NNA, Nω-nitro-L-arginine; NO, nitric oxide; O_2^-, superoxide anion; RCR, respiratory control ratio; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel.

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2003). Impairment in body temperature, wheel-running activity and pain perception has been described in experimental animals associated with this physiopathological state (Brasser and Spear, 2002; Varlinskaya and Spear, 2004). Results from our laboratory demonstrated that 6 h after an acute ethanol exposure, mice exhibited a reduction in motor performance associated with brain cortex mitochondrial dysfunction at the onset of ethanol hangover (Bustamante et al., 2012).

Other researchers reported that melatonin could prevent oxidative damage and mitochondrial DNA (mtDNA) depletion induced by acute ethanol administration (Mansouri et al., 2001). The aim of this work was to study the melatonin effect on motor performance and mitochondrial function during the ethanol hangover in male mice.

EXPERIMENTAL PROCEDURES

Materials

BSA, catalase, cytochrome c, dithiothreitol, EGTA, Folin reagent, glutamate, HEPES, L-arginine, malate, mannitol, Nicotinamide adenine dinucleotide (NADH), Nicotinamide adenine dinucleotide phosphate (NADPH), Nω-nitro-L-arginine (L-NNA), oxyhemoglobin, succinate, superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). The potentiometric probe, 3,3″-dihexyloxacarbocyanine iodide ($DiOC_6$), was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Animals

Swiss mice (*Mus musculus*) weighing 30–40 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 0700 h), 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 Institute of Health (USA), and with the 6344/96 regulation of the Argentinean National Drug Food and Medical Technology Administration (ANMAT). All efforts were made to minimize suffering and reduce the number of animals used.

Methods

Solutions preparation, melatonin exposure and ethanol injections. Ethanol (EtOH), 15% w/v, was prepared by diluting a 95% stock solution of EtOH with 0.9% saline solution (SS). Melatonin (Mel) (Sigma–Aldrich, St. Louis, USA) (purity: 982 mg/g) solution was solubilized in 10% ethanol and mixed in tap water (25 μg melatonin/ml), according to previous research (Kim et al., 2000a,b; Bruno et al., 2005). The final EtOH concentration in melatonin solution was 0.1%. Mice received melatonin solution or its vehicle in drinking water during 7 days during 24 h. At the eighth day, animals received an injection (i.p.) of EtOH (3.8 g/kg BW) or saline (9:00 am). The ethanol 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). In order to ensure the stability of the melatonin solution and properly control its administration to mice, melatonin solution was prepared every day and the bottles containing drinking water were covered to prevent drug degradation by light. Motor performance was evaluated in the early afternoon (3:00 pm) at the onset of ethanol hangover. According to previous researches, ethanol hangover onset was considered 6 h after ethanol injection when blood alcohol concentration (BAC) was close to zero (Bustamante et al., 2012). Melatonin treatment during 7 days before ethanol exposure did not modify BAC levels (data not shown).

Evaluation of melatonin effect on motor performance during ethanol hangover. Motor coordination was evaluated with a modified tightrope test (Boveris and Navarro, 2008). Briefly, the procedure consisted of placing the animal on the middle of a 60-cm long horizontal rope suspended 30 cm above the floor and time was recorded until the animal either reached the end of the rope or fell down during a period of 60 s. A score was assigned accordingly: animals reaching the end of the rope in ⩽6 s were given 1 point and an additional point was given for every additional 6 s needed to complete the test. Animals that stayed on the rope for 60 s without reaching the end were given 11 points. When mice fell down, while the test was running, 11 points were assigned and 1 extra point was added for every 6 s before the test ending time (60 s). The test evaluates the motor performance of the animal as a mean of its intrinsic neuromuscular coordination. For this work, results were shown as a percentage of the motor performance which was calculated considering the maximum score for the test and the score reached for each animal.

Evaluation of melatonin effect on brain cortex mitochondrial function during ethanol hangover

Isolation of mice brain cortex mitochondria. As for the first experiment, animals received melatonin or vehicle during 7 days and, at the eighth day received an i.p. injection of ethanol or saline. Six hours after injection, animals were killed by cervical dislocation in accordance with the directive systems of protection of vertebrate animals for scientific research. Brain cortex was rapidly removed and minced on ice, resuspended in mannitol sucrose HEPES (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. A protease inhibitor cocktail (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM MSF and 1 μg/ml aprotinin) was added to the homogenates and then centrifuged at 600*g* for 10 min at 4 °C. The supernatant was decanted and centrifuged again at 8000*g* for 10 min; the new mitochondrial pellet was washed several times in MSH without EDTA, in order to avoid calcium chelation by this compound. 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 cortex

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