Leptin alters brain adenosine triphosphatase activity in ethanol-mediated neurotoxicity in mice

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ABSTRACT

Introduction: **This study aimed to assess the leptin dependent changes on adenosine triphosphatase (ATPase) activities of the central nervous system during chronic ethanol supplementation.**

Methods: **The mice were divided into four groups. Group 1 consisted of control animals that received isocaloric glucose, Group 2 animals received isocaloric glucose plus exogenous mouse recombinant leptin (230 microgrammes/kilogramme body weight intraperitoneally) every alternate day, Group 3 were alcohol-fed mice (6.32 grammes/kilogramme body weight orally), and Group 4 were alcohol-fed mice that received leptin (230 microgrammes/kilogramme body weight intraperitoneally) every alternate day. The experiment was terminated after giving the mice leptin injections for 15 days.**

Results: **Ethanol feeding for a total period of 45 days (p-value is less than 0.05) significantly elevated the brain lipid hydroperoxide levels and total ATPases, sodium, potassium-ATPase and magnesium-ATPase activities but significantly decreased the calcium-ATPase activity. Leptin injections to ethanol-fed animals further elevated the levels of lipid hydroperoxides, total ATPases, sodium, potassium-ATPase and magnesium-ATPase, while calcium-ATPase activity was reduced significantly.**

Conclusion: **Leptin plays an important role in the pathogenesis of ethanol-induced neurotoxicity by enhancing brain lipid peroxidation and regulating brain ATPase activities in mice. Thus, hyperleptinaemia-induced oxidative stress and enhanced ATPase activities may be important pathogenic factors in brain toxicity.**

Keywords: adenosine triphosphatase, brain, ethanol, leptin, lipid peroxidation, mice, neurotoxicity

Singapore Med J 2006; 47(10):864-868

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INTRODUCTION

Chronic and excessive alcohol abuse is marked by a number of biochemical and physiological changes in the central nervous system (CNS). Some of these changes pertain to alteration of specific neurotransmitter systems[1] and intricate signaling pathways[2]. In spite of over 20 years of experimental and clinical efforts, the mechanism by which ethanol causes neuronal death is not entirely clear. However, a recognised mechanism of ethanol action is its ability to enhance oxidative stress[3]. Due to the presence of high proportions of polyunsaturated fatty acids and low oxidant defence enzymes in the brain, this organ is particularly susceptible to oxidative stress, and free radicals are generated under normal as well as pathological conditions[4]. Therefore, it is possible that oxidative changes exerted by chronic and excessive ethanol consumption may exacerbate the progression of other neurodegenerative disorders. Based on these observations, we had already measured and reported the lipid peroxidation and antioxidant status of the ethanol-treated mouse brain[5]. Our aim in this study was to explore the effect of leptin on the activities of membrane-bound adenosine triphosphatase (ATPase) in the brain of ethanol-fed mice.

Leptin, a 16-kD polypeptide mainly produced by the adipose tissue, exerts its function as a satiety signal by actions in the CNS, mediated by leptin receptors in the arcuate nucleus of the hypothalamus and other brain regions[6]. The co-localisation of leptin and leptin receptors in regions such as the cerebellum, cerebral cortex, and hippocampus suggest that leptin might modulate neural systems, which are distinct from those involved in body weight regulation. Plasma leptin concentration is increased in obese individuals and in animals with diet-induced obesity[7]. Moreover, hyperleptinaemia is associated with systemic and intrarenal oxidative stress and nitric oxide (NO) deficiency, evidenced by reduced urinary excretion of NO metabolites and its second messenger, cyclic guanidine

monophosphate (cGMP)[8]. Recently it was reported that leptin up-regulates renal Na^+, K^+-ATPase by oxidative stress-dependent inactivation of NO[8]. Therefore the objective of the present study was to assess the effect of leptin in ethanol-mediated experimental neurotoxicity in mice.

METHODS

All the animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration number: 166/1999/CPCESA) and animals were cared for in accordance with the Indian National Law on animal care and use. 60 adult (four weeks old) male Swiss albino mice bred and maintained in the local animal house were used in this study. They were housed, three per cage, in plastic cages (47 cm × 34 cm × 18 cm), lined with husk that was renewed every 24 hours, and had free access to drinking water and food. The animals were kept at room temperature (30 ± 2°C) under seminatural light-dark conditions (12 hours light/12 hours dark).

Mouse recombinant leptin (purity >97% as determined by SDS-PAGE & HPLC) was purchased from Sigma Chemical Co (St Louis, MO, USA). Reconstitution of leptin was done by adding 0.5 ml of 0.2 μm-filtered 15 mM HCl. After dissolving the protein, 0.3 ml of 0.2 μm-filtered 7.5 mM NaCl was added. The dissolved protein was then stored in a refrigerator. The hormone was diluted with phosphate-buffered saline (pH 7.4) just before use. Ethanol was obtained from Cuddalore District, South India. All other chemicals used were of analytical grade and the organic solvents were distilled before use.

The animals were divided into four groups. Groups 1 and 2 received a normal diet of standard pellets and isocaloric glucose from a 40% glucose solution. Groups 3 and 4 mice were administered 16% ethanol, 1 ml each (6.32 g kg^{-1} body weight) as an aqueous solution using an intragastric tube daily for 30 days. At the end of this period, the animals were treated as follows for the next 15 days.

Group 1 animals continued to receive the standard pellet diet and isocaloric glucose from a 40% glucose solution daily by intragastric intubation and served as controls. Group 2 animals continued to receive standard pellet diet, isocaloric glucose from a 40% glucose solution and were administered exogenous leptin (230 μg kg^{-1} body weight intraperitoneally) every alternate day. Group 3 animals continued to receive standard pellet diet and 16% ethanol orally. Group 4 animals continued to receive standard pellet diet, 16% ethanol orally and leptin 230 μg kg^{-1} body weight intraperitoneally every alternate day.

The total experimental duration was 45 days, at the end of which the animals were fasted overnight, anaesthetised with an intramuscular injection of ketamine hydrochloride (30 mg kg^{-1} body weight), and sacrificed by cervical dislocation. Brain tissue was removed, cleared of blood, and collected in ice-cold containers containing 0.9% NaCl for the assay of lipid hydroperoxides (LOOH)[9] total ATPases[10], Na^+, K^+-ATPase: EC 3.6.3.9[11], Ca^{2+}-ATPase: EC 3.6.3.8[12] and Mg^{2+}-ATPase: EC 3.6.3.2[13]. The levels of total protein in the brain homogenate was quantified by the same procedure as described Lowry et al[14].

Results were statistically evaluated using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). All the grouped data were statistically determined with the Statistical Package for Social Sciences (SPSS) version 10.0 (Chicago, IL, USA). The differences were considered significant at $p<0.05$.

RESULTS

The levels of LOOH and the activities of total ATPases, Na^+, K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in the brain of control and experimental animals are shown in Table I. Lipid peroxidation indicated by LOOH and the activities of total ATPases, Na^+, K^+-ATPase and Mg^{2+}-ATPase were found to be increased significantly, whereas Ca^{2+}-ATPase activity was significantly reduced in the ethanol-intoxicated mice (Group 3) as compared

Table I. Effect of administering leptin and alcohol on LOOH and ATPases in the mouse brain.

Groups	LOOH (mM/g tissue)	Total ATPase*	Na^+, K^+-ATPase*	Ca^{2+}-ATPase*	Mg^{2+}-ATPase*
Control	96.68 ± 9.70^{ae}	3.68 ± 0.27^{ae}	2.14 ± 0.18^{ae}	2.51 ± 0.22^{ae}	2.26 ± 0.26^{ae}
Control + leptin	97.16 ± 8.11^{be}	3.72 ± 0.23^{be}	2.08± 0.15^{be}	2.48 ± 0.22^{be}	2.31 ± 0.19^{be}
Alcohol	123.1 ± 5.61^{c}	5.03 ± 0.43^{c}	4.05 ± 0.32^{c}	1.60 ± 0.30^{c}	3.54 ± 0.27^{c}
Alcohol + leptin	135.9 ± 4.21^{d}	5.82 ± 0.25^{d}	4.85 ± 0.28^{d}	1.06 ± 0.14^{d}	4.09 ± 0.18^{d}

* μ moles of inorganic phosphorous formed/min/mg protein

Values are mean ± SD for six mice in each group

$^{a-e}$Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

to the normal control mice (Group 1). Administration of exogenous leptin to alcohol-treated mice (Group 4) further elevated the levels of LOOH and the activities of total ATPases, Na^+, K^+-ATPase and Mg^{2+}-ATPase; while the activity of Ca^{2+}-ATPase was found to be lowered as compared to those of the alcohol-fed mice (Group 3). Leptin per se (Group 2) did not alter LOOH, total ATPases, Na^+, K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase significantly.

DISCUSSION

Studies of alcohol-induced brain damage have clearly indicated that alcohol is neurotoxic. The brain is thought to be very susceptible to lipid peroxidation and oxidative injury, and the free radicals produced can easily change the membrane structure[15]. Proper composition of cellular membranes is of great importance to their biological functions. A wide range of membrane functions could be modulated by ethanol through an alcohol-associated increase in the degree of fatty acid unsaturation, a decrease in the total amount of lipids in the cells, and a resulting increase in the membrane fluidity[16].

The brain contains particularly large amounts of polyunsaturated fatty acids and a high content of catalytically-active metal ions, especially in the striatum and hippocampus. Thus, the brain tissue is particularly vulnerable to membrane lipid peroxidation that disturbs fundamental functions of the brain. Results from the current study support the suggestion that chronic ethanol consumption leads to important changes in the brain, evidenced by an increase in lipid peroxidation products, such as LOOH. Moreover, findings from numerous studies have shown that lipid peroxidation may be implicated in the irreversible loss of neuronal tissue after brain or spinal cord injury as well as in degenerative neurological disorders. The damage of nerve endings by peroxidation products may lead to large changes in the transport of neurotransmitters resulting in an alteration of the function of the CNS[17].

Administration of exogenous leptin further significantly enhances the brain LOOH. Several in-vivo studies have demonstrated that leptin stimulates reactive oxygen species (ROS) production by inflammatory cells[18], endothelial cells[19] and other cell types[20]. In contrast, little is known about the effect of leptin on antioxidant mechanisms. Recently, we have also demonstrated that experimental hyperleptinaemia induced in mice by administration of exogenous leptin induces systemic oxidative stress and decreased antioxidant enzyme activity in the brain[5]. However, the causal relationship between two effects remain unclear.

The membrane-bound enzymes such as Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes at the expense of ATP hydrolysis. ROS are proposed to be involved in alcohol-induced pathology and proteins, in general, and enzymes such as Ca^{2+}-ATPase are very sensitive to oxidative damage[21]. Membrane lipid peroxidation results in altered membrane fluidity[22], altered permeability to ions[23] and changes in the activities of membrane-bound enzymes[24]. Ca^{2+}-dependent ATPase, the enzyme responsible for active calcium transport, is known to be inhibited due to membrane lipid peroxidation in different types of muscles[25] and red blood cells[26]. Changes in neuronal calcium levels may be an important aspect of ethanol neurotoxicity[27]. In our study, ethanol and leptin inhibited Ca^{2+}-ATPase activity in the mouse brain. The decreased activity of Ca^{2+}-ATPase observed during leptin and alcohol administration may be due to depletion of reduced glutathione or increased lipid peroxidation, which inhibits thiol-dependent enzymes[5].

Na^+, K^+-ATPase is a crucial enzyme responsible for the active transport of sodium and potassium ions in the CNS necessary to maintain the ionic gradient for neuronal excitability. It is well known that Na^+, K^+-ATPase is involved in the restoration and maintenance of sodium and potassium equilibrium through neuronal membranes both at rest and after passage of a nerve impulse[28,29]. Na^+, K^+-ATPase is highly concentrated in synaptic nerves endings, and regulatory mechanisms that control this enzymatic activity are essential in maintaining metabolic activity of the synaptic region and in processes directly related to neurotransmission[29]. Na^+, K^+-ATPase has, for many years, been a logical candidate as a site of action for alcohol, and as a basis for broader biological effects of alcohol. Because many signs and symptoms of alcohol intoxication are due to changes in the CNS function, it would be logical to suspect that the Na^+, K^+-ATPase may be involved in, or a basis for, some of those effects[30]. Many mechanisms are involved in the neurochemical events underlying effects of ethanol and development of ethanol addiction, including changes in the Na^+, K^+-ATPase. The effects of ethanol may be associated with both inhibition and activation of Na^+, K^+-ATPase/Na/K-pump depending on the dose, route of administration, and length of exposure[31].

Foley and Rhoads have demonstrated that in rat cerebral cortex synaptosomes, stimulatory effect of ethanol on the Na^+, K^+-ATPase is related to its action

on ouabain-sensitive (alpha-2 and alpha-3) rather than relatively ouabain-resistant (alpha-1) Na^+, K^+-ATPase isoforms. Ethanol induces sodium pump activation possibly via antagonising the effect of an unidentified Na^+, K^+-ATPase endogenous inhibitory factor[32]. Additionally, chronic in-vivo ethanol treatment may induce the up-regulation of brain Na^+, K^+-ATPase[33]. Our results are in agreement with evidence presented by above-mentioned and other investigators, showing that Na^+, K^+-ATPase activity is increased in an experimental model of alcohol-mediated neurotoxicity in mice[33,34], a fact that may be an important factor in the pathogenesis of metabolic complications of the CNS. However, the physiological significance of these alterations of brain Na^+, K^+-ATPase in alcohol-induced hepatotoxicity remains to be clarified.

Exogenous leptin administration further elevated the activity of Na^+, K^+-ATPase in the brain of ethanol-intoxicated mice. Interestingly, the OB-R in the hypothalamus appears to have a long intracellular domain of 302 aa (long form or OB-Rb)[6]. The long intracellular domain of the OB-Rb contains putative motifs Janus protein activating kinase (JAK) and signal transducers and activators of transcription (STATs) that are not present in the OB-Ra[35]. The OB-Rb can activate the STAT proteins in the hypothalamus after in-vivo leptin administration in lean but not in db/db mice[36]. However, other isoforms of the leptin receptor such as OB-Ra, which trigger rapid nongenomic signaling mechanisms, are abundantly expressed in the kidney[37]. Interestingly, Na^+, K^+-ATPase activity is increased in the renal cortex and medulla of rats with diet-induced obesity[7], but unchanged[38] or increased only in the renal cortex[39] in obese Zucker rats which have a mutation of the leptin receptor gene, suggesting that functional leptin signaling is necessary for obesity-induced up-regulation of renal sodium pump. In the present study, we have observed significantly increased brain Na^+, K^+-ATPase activity in ethanol-fed mice after leptin administration, which may be due to increased signaling of the leptin receptor in the brain.

Mg^{2+}-ATPase plays a significant role in Mg^{2+} homeostasis maintaining high brain intracellular Mg^{2+} content and can modulate the activity of Mg^{2+}-dependent enzymes. In our study, mouse brain Mg^{2+}-ATPase and total ATPases activities were found to be remarkably elevated on ethanol supplementation. These results are in agreement with those of Israel and Kuriyama[40] who assayed Mg^{2+}-ATPase in whole liver homogenate. On administering leptin, the levels of these enzymes were further significantly increased.

In conclusion, our earlier studies confirm that leptin elevated ethanol-induced neurotoxicity[5]. Further, we had demonstrated that plasma leptin levels were significantly increased in ethanol-fed mice (unpublished data). Herein, we have observed that co-administration of leptin along with ethanol could increase the oxidative stress and upregulate the brain ATPase activities. Collectively, these findings indicate that leptin plays an important role in the pathogenesis of ethanol-induced neurotoxicity in mice. Thus, hyperleptinaemia as well as leptin resistance and/or leptin induced oxidative stress may be important pathogenic factors.

ACKNOWLEDGEMENT

This work was financially supported by the Indian Council of Medical Research (ICMR), New Delhi, India in the form of a Senior Research Fellowship to Dr V Balasubramaniyan.

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