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EXPERIMENTAL STUDY

Effects of ethanol withdrawal on the activity of rho-kinase in rat brain

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ABSTRACT

Besides its effect regarding addiction, ethanol also damages the central nervous system when it is used at high doses for a long time. The increase in the activity of Rho/Rho kinase pathway leads to central nervous system pathologies such as cerebral injury and epileptogenesis. The aim of this study was to investigate the contribution of Rho/Rho Kinase pathway to the degenerative and addictive effects of ethanol. For this purpose, we determined the Rho-kinase activity in striatum and hippocampus of rat brain. Wistar rats were treated with ethanol in a special liquid diet for 21 days. An isocaloric liquid diet without ethanol was given to the rats in the control group during the study. At the end of the 21 day ethanol exposure, one group was kept on taking ethanol and another group was withdrawn from ethanol. The rats were decapitated and their brains were taken out. Striatum and hippocampus were isolated. Phospho-moesin protein levels were measured in striatum and hippocampus homogenates using by Western blot analysis. The Rho-kinase (ROCK) activity in the striatum was found to be significantly decreased in ethanol exposed rats. In the hippocampus, there was a significant increase in the ROCK activity in the ethanol group. Our results indicated that ethanol caused some significant changes in Rho/Rho Kinase pathway in rat brain *(Fig. 2, Ref. 25)*. Text in PDF *www.elis.sk.*

KEY WORDS: ethanol, Rho-kinase, hippocampus, striatum, rat.

Introduction

Ethanol abuse and dependence remain among the most common substance abuse problems worldwide. Ethanol is a poison with direct toxic effects on nerve and muscle cells. Alcohol-related neurologic diseases include Wernicke–Korsakoff disease, alcoholic cerebellar degeneration, alcoholic myopathy, alcoholic neuropathy, foetal alcohol syndrome, alcohol withdrawal syndrome with seizures and delirium tremens. The discontinuation of chronic administration of ethanol is associated with excitatory withdrawal signs called ethanol withdrawal syndrome. The signs of ethanol withdrawal syndrome in humans (Thompson, 1978) and rodents (Majchrowicz, 1975) have been well determined the mechanisms underlying physical dependence to ethanol and ethanol withdrawal syndrome are poorly understood.

Rho-kinase (ROCK) contributes to several physiological processes by modulating cell migration, proliferation and survival, axonal outgrowth, dendrogenesis, endocytosis and tumour metastasis. Abnormal activation of the Rho/ROCK pathway has been observed in various disorders of the central nervous system (CNS) (Mueller et al, 2005), and it is important in the pathogenesis of several neurological diseases such as spinal cord injury, stroke, multiple sclerosis and neuropathic pain (Mueller et al, 2005).

The inhibition of ROCK by the ROCK inhibitors improved mechanical hypoalgesia and motor dysfunction caused by spinal canal stenosis (Ito et al, 2007), protected against ischemia-induced delayed neuronal death when treatment was started 24 h after ischemia (Satoh et al, 2008), decreased tumour cell metastasis and invasion (Kamai et al, 2003; Lepley et al, 2005). The Rho family proteins RhoA, Rac1, and Cdc42 have been implicated in the phosphorylation of moesin in various cell lines. The activation of RhoA, Rac1, or Cdc42 is involved in the phosphorylation of moesin. In neuronal cells, moesin is highly expressed in PC12 cells and glioma cells, whereas the three ERM proteins are equally expressed in astrocytes (Songhee et al, 2008).

The aim of the present study was to investigate the contribution of Rho/Rho-kinase pathway to ethanol withdrawal. The regions of the brain named as striatum and hippocampus are related with ethanol addiction and withdrawal. We measured the amount of p-moesin as an indicator of the Rho-kinase activity in these regions.

Methods

Animals and laboratory

All procedures in this study are in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the

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National Institutes of Health. Local ethical committee approval was also attained. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experimental subjects were adult male Wistar rats (266–278 g weight at the beginning of the experiments). They were housed in a quiet and temperature and humidity-controlled room (22 ± 3 °C and 65 ± 5 %, respectively) in which a 12-h light/dark cycle was maintained (07:00–19:00 h light). Exposure to ethanol and all behavioral experiments involved in ethanol withdrawal syndrome were carried out in the separate and isolated laboratories, which have the same environmental conditions with the colony room.

Chronic exposure to ethanol

The rats were housed individually and ethanol was given in the modified liquid diet for chronic ethanol exposure as previously described (Uzbay et al, 1995). They received a modified liquid diet with or without ethanol ad libitum. No extra chow or water was supplied. The composition of the modified liquid diet with ethanol is as follows: cow's milk 925 ml (Danone, Turkey), 25–75 ml ethanol (96.5 % ethyl alcohol; Tekel, Turkish State Monopoly), sucrose 17 g. This mixture supplies 1000.7 kcal/l. At the beginning of the study, rats were given the modified liquid diet without ethanol for 7 days. Then liquid diet with 2.4 % ethanol has been administered for 3 days. The ethanol concentration was increased to 4.8 % during the following 4 days and finally to 7.2 % for 21 days. Liquid diet was freshly prepared daily and presented at the same time of the day (10: 00 h). The weight of the rats was recorded every day, and daily ethanol intake was measured and expressed as grams per kilograms per day. Control rats (n = 3) were pair fed an isocaloric liquid diet containing sucrose as a caloric substitute to ethanol.

Evaluation of ethanol withdrawal syndrome

At the end of the exposure to 7.2 % ethanol-containing liquid diet, ethanol was removed from the diet. First group was kept on receiving ethanol and the other group was exposed to ethanol withdrawal.

Western blot analysis

All the rats which received ethanol, those exposed to ethanol withdrawal and exposed to an audiogenic stimulus were decapitated at 21st days. Brains were removed and corpus striatum and hippocampus were dissected within 5 min on a block of ice. Corpus striatum and hippocampus was homogenized with lyses buffer solution, (composition: Tris-HCl (pH 7.4) 50 mM, NaCl 400 mM, EGTA 2 mM, EDTA 1 mM, dithiothreitol 1 mM, phenylmethylsulphonyl fluoride 10 μM, leupeptin 1 μg/mL, pepstatin 1μg/mL, benzamidine 1 mM). The homogenate was centrifuged at 13000 g for 10 min at 4 °C and the supernatant was removed. It was then used for protein analysis (Bradford method) and Western blot analysis. Equal amounts of proteins (50 μg) were loaded in wells, electrophoresed on 8 % polyacryamide-SDS gels and then transferred to a nitrocellulose membrane overnight. The membrane was blocked with the blocking agent of an enhanced chemiluminescence kit (ECL plus; Amersham Biosciences, Freiburg, Germany) in Tris-buffered solution containing 0.05 % Tween-20 for 1 h. It was then probed with primary antibodies raised against p-moesin (Polyclonal IgG, Santa Cruz Biotechnology Inc., CA, U.S.A.) at 1 : 200 dilution, followed by a horseradish peroxidase-conjugated secondary antibody (donkey anti-goat, 1 : 1000, Santa Cruz Biotechnology Inc.). Protein blots were then detected with the plus chemiluminescence detection kit (Amersham Biosciences) and visualized on commercial X-ray film.

Statistical analysis

All data represent the mean ± standard error of the mean (S.E.M.) of n observations. For statistical comparison, one way analysis of variance (ANOVA) followed by the Bonferroni post hoc test or Student's t test, if appropriate, was used. The P value lower than 0.05 was considered significant.

Results

Phosphorylation of Moesin at Thr-558 in the Hippocampus and Striatum

In hippocampus, there was an increase in the ROCK activity in the group taking ethanol ($p < 0.05$) and no change in the other group that was exposed to ethanol withdrawal (Fig. 1). The ROCK activity in the striatum decreased significantly ($p < 0.01$) in the

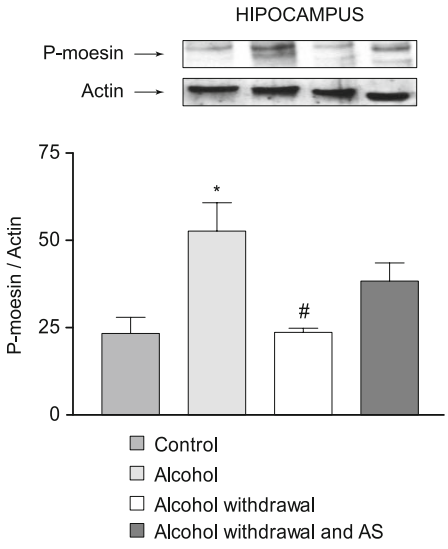

Fig. 1. Hippocampal p-moesin protein expression was determined by Western blotting. The homogenates were submitted to sodium dodecyl sulphate (SDS)-PAGE with 8 % polyacrylamide and then transferred to a nitrocellulose membrane (0.22 μm, Santa Cruz). The membrane was blocked with the blocking agent of the enhanced chemiluminescence advance kit (ECL plus) in Tris-buffered solution containing 0.05 % Tween- 20 (TBS-T) for 1 h. It was then probed with a primary antibody raised against p-moesin (Polyclonal IgG, Santa Cruz) at 1 : 200 dilution, followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1 : 1000). Actin was used as loading control. Blots were then assayed with an Enhanced Chemiluminescence Detection Kit (ECL plus, Amersham Bioscience), and visualized on commercially available X-ray films (* vs control; $p < 0.05$, # vs alcohol; $p < 0.05$).

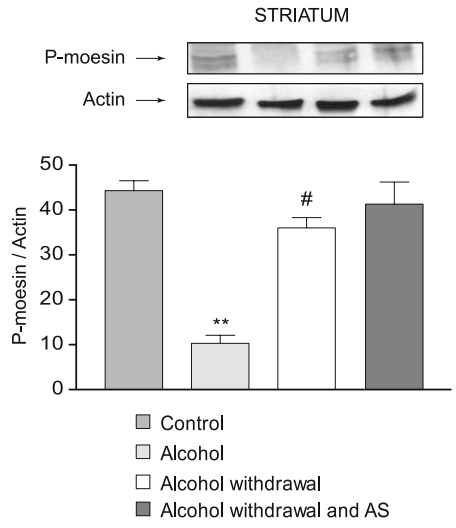

Fig. 2. Striatal p-moesin protein expression was determined by Western blotting. The homogenates were submitted to sodium dodecyl sulphate (SDS)-PAGE with 8 % polyacrylamide and then transferred to a nitrocellulose membrane (0.22 µm, Santa Cruz). The membrane was blocked with the blocking agent of the enhanced chemiluminescence advance kit (ECL plus) in Tris-buffered solution containing 0.05 % Tween- 20 (TBS-T) for 1 h. It was then probed with a primary antibody raised against p-moesin (Polyclonal IgG, Santa Cruz) at 1 : 200 dilution, followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1 : 1000). Actin was used as loading control. Blots were then assayed with an Enhanced Chemiluminescence Detection Kit (ECL plus, Amersham Bioscience), and visualized on commercially available X-ray films (vs control; $p < 0.01$, # vs alcohol; $p < 0.05$).**

group taking ethanol and increased ($p < 0.05$) in another group where ethanol was removed (Fig. 2).

Discussion

This is the first study that determines the connection between alcohol withdrawal and Rho/Rho-kinase pathway. The main result obtained from the present study was that the ROCK activity in the striatum decreased significantly in the group taking ethanol and increased in the group that ethanol was removed. In hippocampus, there was an increase in the ROCK activity in the group taking ethanol and no change in the group that was exposed to ethanol withdrawal. These changes in the ROCK activity may play a role in the symptoms caused by ethanol withdrawal. Dopamin and serotonin may be responsible for the changes in the ROCK activity. Recent studies determined that haloperidol, a nonspecific dopaminergic antagonist, increased stereotyped behavior during the same period of ethanol withdrawal in rats (Uzbay et al, 1994). In addition, it was seen that serotonin re-uptake inhibitors lowered the symptoms.

Synaptic plasticity is dependent on the structural regulation of the actin cytoskeleton in dendritic spines (Carlisle et al, 2005; Schuber et al, 2007; Sekino et al, 2007). Sekino et al (2007) and Schubert and Dotti (2007) have reviewed in detail the molecular players involved in the maintenance of the synaptic cytoarchitecture in dendritic spines, such as the regulation of F-actin cytoskeleton through the various actin-binding proteins and the molecular regulators of actin dynamics, i.e. membrane receptors and downstream signalling cascades. Rho GTPases have emerged as the key regulators in the control of actin filament assembly in the dendritic spines (Schuber et al, 2007; Sekino et al, 2007). The Rho family GTPases including RhoA, Rac1, and CDC42 are the major regulators in synaptic plasticity, both in dendrite morphogenesis and stability as well as in growth cone motility and collapse (Lin et al, 2007). Small Rho proteins act as molecular switches between inactive GDP-bound and active GTP-bound forms under the regulation of several Rho GEFs (guanine nucleotide exchange factors) and Rho GAPs (GTPase activating protein) (Linseman et al, 2008; Schiller, 2006). In neurons, there are several upstream regulators of Rho GTPases, such as NMDA, ephrin-B, and semaphorin 3A receptors as well as a number of Rho GEFs, which can activate the Rho proteins and in that way subsequently modulate several downstream effector proteins (Lim et al, 2008; Kasri et al, 2008) Kalirin-7, and Tiam1 are well-known Rho GEFs, which can regulate the morphogenesis of dendritic spines. RhoA, Rac1, and Cdc42 proteins are linked to distinct upstream and downstream regulators. In many instances, RhoA, and Rac1/Cdc42 have antagonistic effects on dendritic plasticity, this being mostly due to their different effector molecules. RhoA-GTP activates the Rho-kinases (ROCK1, and ROCK2) and ROCK2 is highly expressed in the brain.

To evaluate the in vivo functions of CNS ROCKs, inhibitors have been administered in several model systems. Beneficial neurological effects were observed in animal models of Alzheimer's disease, neuropathic pain, demyelinating/inflammatory diseases, stroke and spinal-cord injuries (Bito et al, 2000; Zhou et al, 2003; Eldawoody et al, 2010). Further analysis of these pathways is likely to accelerate molecular understanding in neuroscience and provide the potential for novel therapeutics in neurology.

Glutamatergic system and NMDA receptors have been proposed to have a crucial role in the development of the ethanol withdrawal signs (Krystal et al, 2003). Glutamate is an excitatory neurotransmitter, which is responsible for brain damage and also for epileptogenesis, activates Rho-kinase in neuronal cells (Jeon et al, 2002). Furthermore, it has been recently demonstrated that the Rho/Rho-kinase signalling pathway is upregulated and may be involved in NMDA-induced retinal neurotoxicity, because fasudil, a Rho-kinase inhibitor, was found to be neuroprotective against glutamate-related excitotoxicity (Kitaoka et al, 2004). NMDA leads to an increase in retinal RhoA protein level and activates Rho-kinase, causing neuronal toxicity. Inhibition of Rho-kinase prevented glutamate induced neurotoxicity. Moreover, in cultured hippocampal neurons as well as in whole-brain synaptosomal fractions, RhoA was associated with glutamate receptors at the plasma membrane of dendritic spines (Schubert et al, 2006). These findings, although not directly linking ROCK action as the underlying mechanism for neuron damage, are suggestive of this connection since the inhibition of ROCK may be involved in ethanol withdrawal syndrome.

In conclusion, our results suggest that Rho-kinase activity in some areas of rat brain is influenced by ethanol consumption and withdrawal. The drugs that affect on brain Rho-kinase system may be effective in the treatment of alcohol withdrawal syndrome.

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