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Ethanol-mediated inhibition of mitogen-activated protein kinase phosphorylation in mouse brain

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Abstract

Ethanol (1.5–3.5 g/kg body weight) was administered intraperitoneally to mice and the phosphorylation of MAP (mitogen-activated protein) kinase in the cerebral cortex was determined using phospho-specific MAP kinase antibodies. Ethanol inhibited the phosphorylation of MAP kinase in a dose- and time-dependent manner. Developmental studies demonstrated that the levels of phospho-MAP kinase increased from fetal cortex (prenatal) to 16-day-old mice (postnatal) and remained constant up to 4 months of age. However, ethanol (3.5 g/kg) decreased the phospho-MAP kinase staining in all of the age groups studied. Subcellular fractionation studies demonstrated that ethanol inhibited the phosphorylation of MAP kinase in both the cytosol as well as nucleus, but did not alter the levels of MAP kinase. Likewise, MK-801 (0.4 mg/kg) or flurazepam (75 mg/kg) also decreased the phospho-MAP kinase content. These data indicate that ethanol may inhibit the phosphorylation of MAP kinase in vivo by either inhibiting NMDA receptors or activating GABA receptors.

Keywords: Ethanol; MAP (mitogen-activated protein) kinase; Cerebral cortex

1. Introduction

Ethanol intoxication is associated with deleterious effects on the brain that include the inhibition of the NMDA receptors (Lovinger et al., 1989), voltage-dependent Ca^{2+} channels (Skattebol and Rabin, 1987) and potentiation of GABA receptor function (Mehta and Ticku, 1999). A variety of studies have demonstrated the involvement of NMDA receptors in the generation of long-term potentiation. Loss of memory and learning abilities are the result of ethanol's action on the central nervous system. Understanding the biochemical mechanisms that underlie alcohol's ability to inhibit long-term potentiation may lead to new treatments for alcoholism. Ca^{2+} influx through either voltage sensitive Ca^{2+} channels (Rosen et al., 1994) or glutamate receptor (Sala et al., 2000) leads to the activation of Ras and MAP (mitogen-activated protein) kinase signaling cascade. MAP kinase(s) play an important role in transducing the extracellular signals into the nucleus following stimulation by neurotransmitters and/or growth factors. Several studies have provided evidence for a role of MAP kinases in the cell growth (Mii et al., 1996), differentiation (Sale et al., 1995) and long-term memory (Kornhauser and Greenberg, 1997). A typical MAP kinase signaling pathway consists of a MAP kinase, a MAP kinase kinase and an activator of MAP kinase kinase (MAP kinase kinase kinase) that transmit signals by sequential phosphorylation. An ethanol-induced inhibition of MAP kinase activity has been reported earlier (Hendrickson et al., 1998). Another study, however, demonstrated the potentiation of MAP kinase by ethanol (Chen et al., 1998a). We have previously shown that acute ethanol treatment can inhibit the phosphorylation of MAP kinase in cultured cortical neurons (Kalluri and Ticku, 2000). In the present study, we demonstrate the inhibition of MAP kinase phosphorylation by ethanol in vivo. We used MAP kinase/ERK1/2 (extracellular signal regulated kinase) interchangeably.

2. Materials and methods

2.1. Animals and tissue preparation

C57BL/67CR mice were purchased from Harlan (Indianapolis, IN) and housed in a room maintained at a constant temperature of 22 ± 1 °C with free access to food and water and kept on a 12-h light/12-h dark cycle. The protocol used

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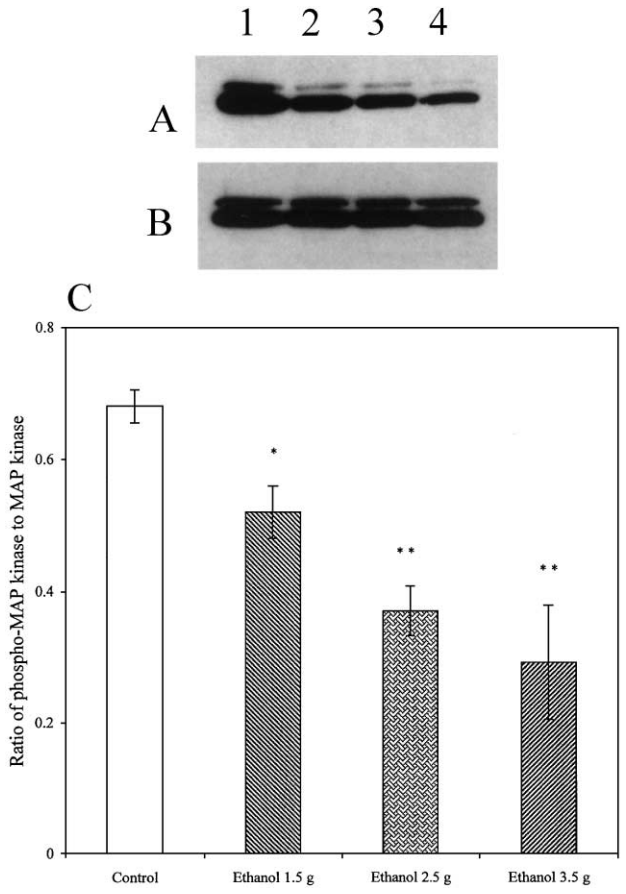

Fig. 1. The effect of varying concentrations of ethanol on the phosphorylation of MAP kinase. Ethanol (1.5, 2.5, 3.5 g/kg body weight) was injected into mice, sacrificed after 10 min and cortex processed as described in Section 2. Thirty micrograms of lysate was subjected to Western blotting and probed with phospho-MAP kinase and MAP kinase antibodies. The data are represented as means ± S.E. of four to five experiments and analyzed by Students *t*-test. * $P < 0.01$ as compared to control. (A) Phospho-MAP kinase; (B) MAP kinase; (C) ratio of phospho-MAP kinase to MAP kinase. (1) Control; (2) ethanol, 1.5 g/kg body weight; (3) ethanol, 2.5 g/kg body weight; (4) ethanol, 3.5 g/kg body weight.

to administer ethanol was described earlier (Miyakawa et al., 1997). Briefly, absolute ethanol was diluted in saline (25% w/v) and injected (3.5 g/kg body weight) intraperitoneally into mice (age 3–4 months) unless otherwise stated. Likewise, MK-801 (0.4 mg/kg) and flurazepam (75 mg/kg) were dissolved in saline and injected into mice. Mice were sacrificed following drug administration and the cerebral cortex homogenized in lysis buffer (20 mM Na_2HPO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 2% Triton X-100, 1 mM Na_3VO_4, 0.5% deoxycholate, 1 mM phenyl methyl sulfonyl fluoride and 1 mg/ml bacitracin), centrifuged at 13,000*g* in an eppendorf centrifuge for 10 min to clarify lysate.

2.1.1. Subcellular fractionation

Cerebral cortex was homogenized in 0.32 M sucrose made in (20 mM Na_2HPO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenyl methyl sulfonyl fluoride and 1 mg/ml bacitracin). The homogenate was centrifuged at 1000*g* for 10 min to separate crude nuclei and the supernatant was used as cytosol. All of the experimental procedures were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. Electrophoresis and immunoblotting

After homogenizing the tissue in lysis buffer, 30 μg of protein (Lowry et al., 1951) was precipitated with cold acetone at −80 °C for 1 h. The precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel

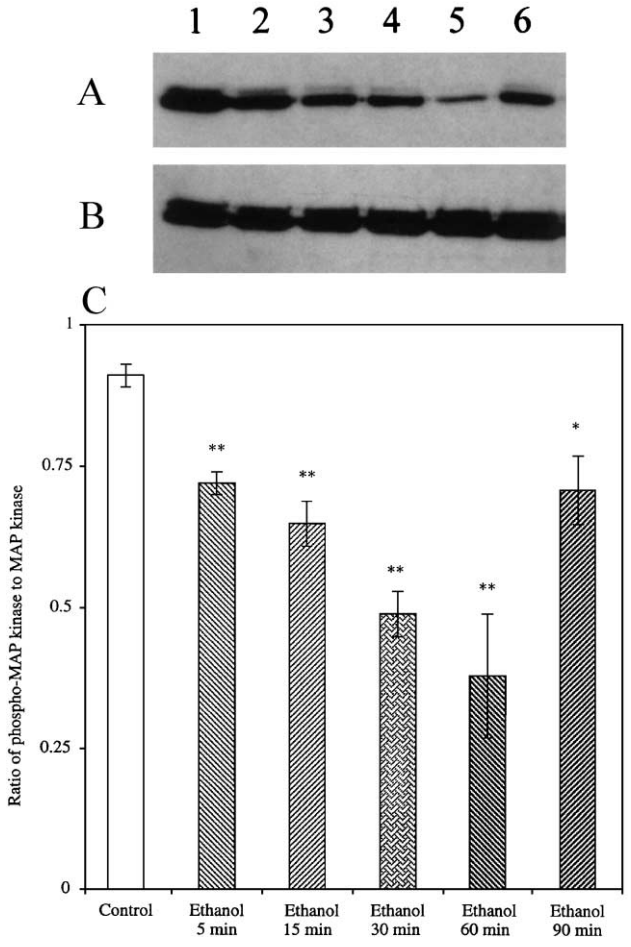

Fig. 2. The effect of acute ethanol administration on the phosphorylation of MAP kinase. Ethanol (3.5 g/kg body weight) was injected intraperitoneally and the animals were sacrificed at different time intervals. The cortex was homogenized in lysis buffer as described in Section 2. Thirty micrograms of lysate was subjected to Western blotting and probed with phospho-MAP kinase and MAP kinase antibodies. The data are represented as means ± S.E. of six experiments and analyzed by Students *t*-test. * $P < 0.05$, ** $P < 0.01$ as compared to control. (A) Phospho-MAP kinase; (B) total MAP kinase; (C) ratio of phospho-MAP kinase to MAP kinase. (1) Control; (2) ethanol (5 min); (3) ethanol (15 min); (4) ethanol (30 min); (5) ethanol (60 min); (6) ethanol (90 min).

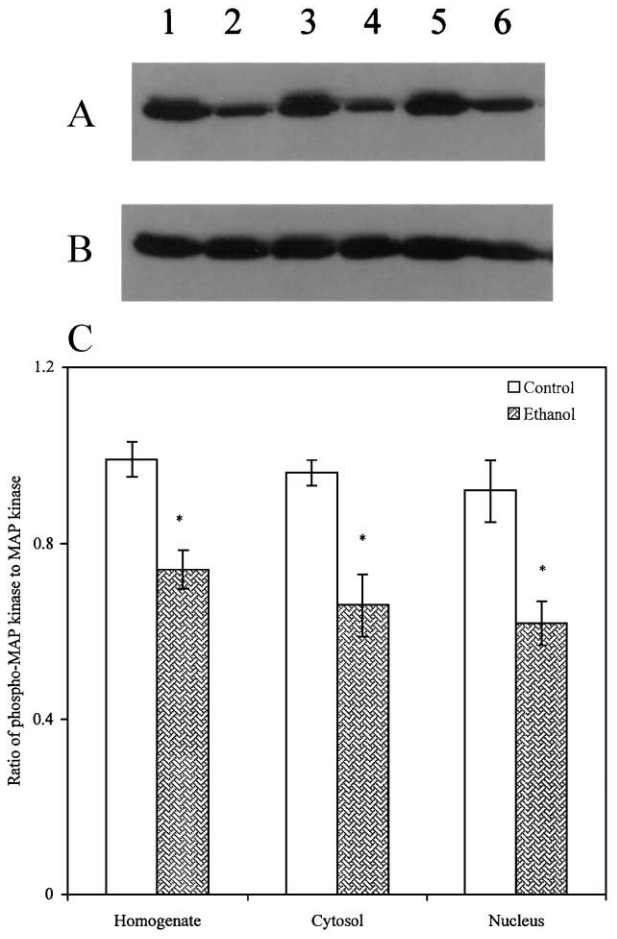

Fig. 3. Effect of ethanol on the subcellular distribution of phospho-MAP kinase. Mice were injected with ethanol (3.5 g/kg body weight), sacrificed after 10 min and cortex was homogenized in 0.32 M sucrose made in (20 mM Na_2HPO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenyl methyl sulfonyl fluoride and 1 mg/ml bacitracin). The homogenate was centrifuged at 1000*g* to separate crude nuclei and the supernatant was used as cytosol. Thirty micrograms of crude cytosol and nuclei were subjected to Western blotting and probed with phospho-MAP kinase and MAP kinase antibodies. The data are represented as means ± S.E. of eight experiments and analyzed by ANOVA. * $P < 0.01$ as compared to respective controls. (A) Phospho-MAP kinase; (B) MAP kinase; (C) ratio of phospho-MAP kinase to MAP kinase. (1) Control homogenate; (2) ethanol homogenate; (3) control cytosol; (4) ethanol cytosol; (5) control nuclei; (6) ethanol nuclei.

electrophoresis (SDS-PAGE) (Laemmli, 1970) and transferred to polyvinylidene difluoride membrane (Towbin et al., 1979). The membrane was blocked with 5% milk/0.1% Tween 20 in Tris-buffered saline for 1 h followed by overnight incubation with primary antibody phospho-ERK1/ERK2 (1:2000) or p42/44 MAP kinase (1:1000) (New England Biolabs). After several washes, peroxidase-coupled secondary antibody (anti-mouse IgG/anti-rabbit IgG; New England Biolabs) was added and incubated for 1 h. The membrane was washed and specific bands were visualized using super signal detection reagents (Pierce Rockford, IL).

2.3. Data analysis

Immunoblot band intensity was measured by NIH image system (Mhatre and Ticku, 1992). Data was analyzed by analysis of variance (ANOVA) supplemented with Duncan's multiple range test or Students *t*-test. Differences were considered to be statistically significant when $P < 0.05$. The values are represented as means ± S.E. of four to eight individual experiments.

3. Results

To determine the phosphorylation of MAP kinase (ERK 1/2), we used phospho-specific antibodies and normalized the data by probing with nonphosphorylated ERK 1/2. The results are summarized as the ratio of phospho-specific ERK to total ERK. In preliminary experiments, varying doses of ethanol were used to study the dose response relationship of ethanol on phospho-MAP staining. Intraperitoneal injection

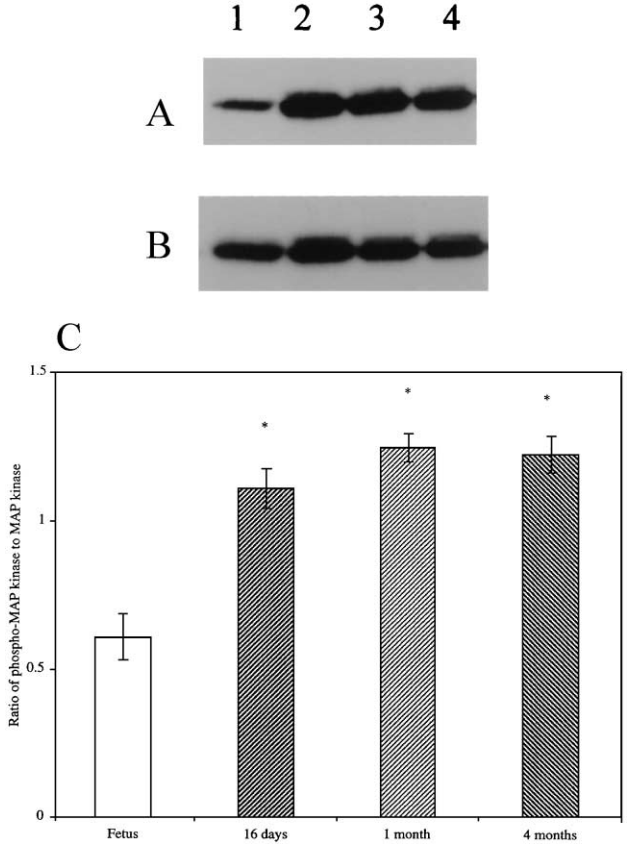

Fig. 4. Phosphorylation of MAP kinase as a function of development. Mice of different age groups were sacrificed and processed as described in Section 2. Thirty micrograms of lysate was subjected to Western blotting and probed with phospho-MAP kinase and MAP kinase antibodies. The data are represented as means ± S.E. of seven experiments and analyzed by ANOVA. * $P < 0.01$ as compared to fetal cortex. (A) Phospho-MAP kinase; (B) MAP kinase; (C) ratio of phospho-MAP kinase to MAP kinase. (1) Fetal cortex; (2) 16-day-old mice; (3) 1-month-old mice; (4) 4-month-old mice.

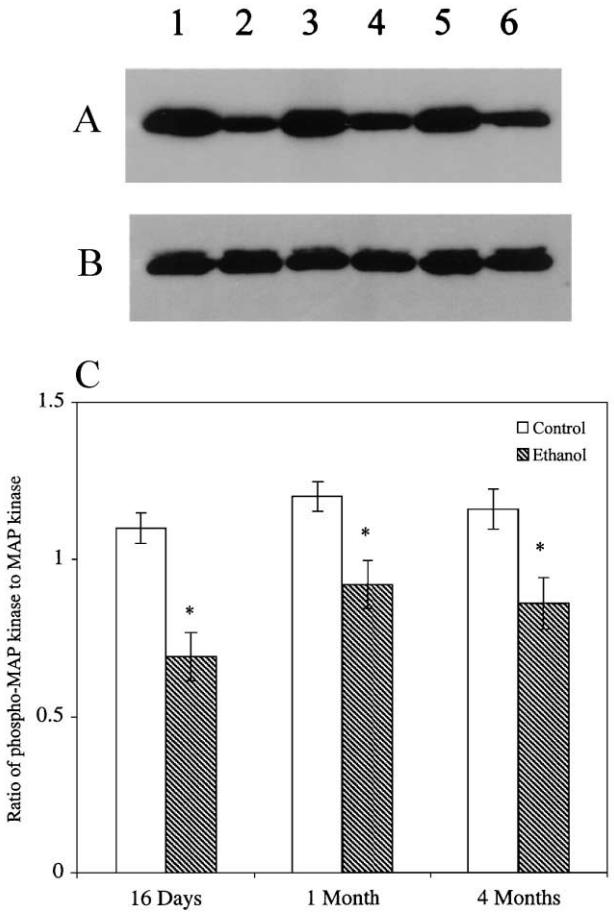

Fig. 5. Effect of ethanol (3.5 g/kg body weight) on the phospho-MAP kinase staining in mice of different age groups. Mice were injected with ethanol and cortices were processed as described in Fig. 1. Thirty micrograms of lysate was subjected to Western blotting and probed with phospho-MAP kinase and MAP kinase antibodies. The data are represented as means ± S.E. of 10 experiments and analyzed by ANOVA. * $P < 0.01$ as compared to respective controls. (A) Phospho-MAP kinase; (B) MAP kinase; (C) ratio of phospho-MAP kinase to MAP kinase. (1) Control, 16 days old; (2) ethanol, 16 days old; (3) control, 1 month old; (4) ethanol, 1 month old; (5) control, 4 months old; (6) ethanol, 4 months old.

of ethanol (1.5, 2.5, 3.5 g/kg body weight) decreased the phosphorylation of MAP kinase (Fig. 1). Ethanol at 3.5 g/kg body weight produced a higher inhibition. This concentration was used in subsequent experiments. We next examined the time course (5, 15, 30, 60 and 90 min) of MAP kinase phosphorylation following intraperitoneal administration of ethanol (3.5 g/kg body weight). Results indicate that the phosphorylation of MAP kinase decreased starting from 5 to 60 min of time after which it was reverting towards control levels (Fig. 2). There was no change in the total MAP kinase levels, demonstrating a specific decrease in the phosphorylation of MAP kinase. MAP kinase, once phosphorylated in the cytosol, is translocated into the nucleus. We studied the subcellular distribution of phospho-MAP kinase following ethanol administration. Results show that ethanol decreased the phospho-MAP kinase levels in both the cytosol as well as nucleus (Fig. 3). The total MAP kinase remained the same, indicating that acute ethanol decreases net phosphorylation without effecting the translocation of MAP kinase.

To further test the effect of ethanol on development, we used mice of different ages ranging from fetal cortex to 4-month-old animals. The developmental profile revealed that the phospho-MAP kinase content increased from fetal brain to 16-day-old mice indicating a spurt in the phosphorylation and remained the same thereafter up to 4 months of age (Fig. 4). Intraperitoneal injection of ethanol (3.5 g/kg body weight) decreased the phosphorylation of MAP kinase in all the three age groups studied (16 days, 1 month and 4 months) (Fig. 5). The magnitude of inhibition was the same in the three groups of animals.

Like ethanol, MK-801 (0.4 mg/kg) and flurazepam (75 mg/kg) also decreased the phosphorylation of MAP kinase in the brain following their administration (Fig. 6).

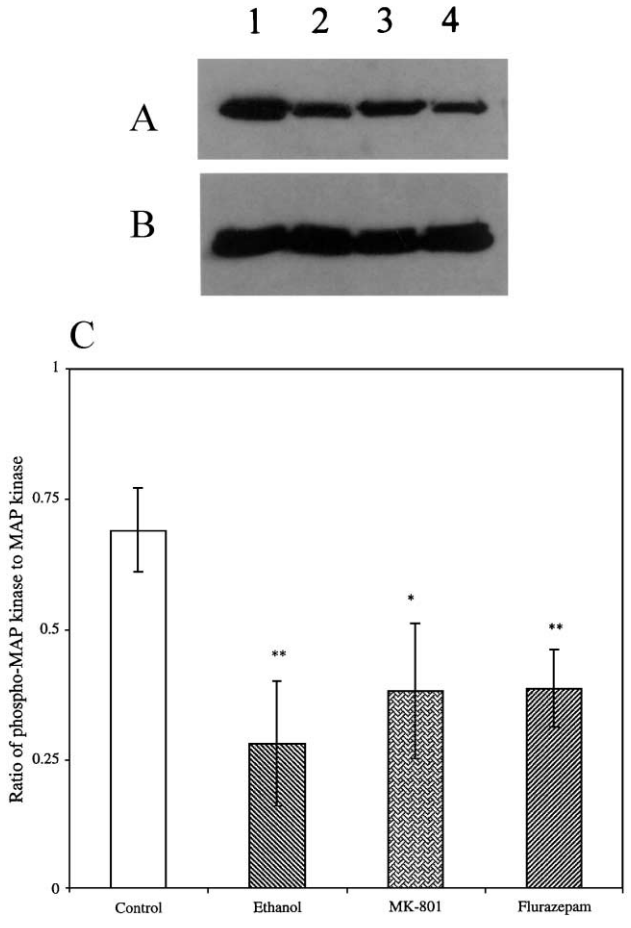

Fig. 6. Effect of ethanol (3.5 g/kg), MK-801 (0.4 mg/kg) or flurazepam (75 mg/kg) on the phospho-MAP kinase content. Mice were injected with drugs and cortical tissue was processed as in Fig. 1. The data are represented as means ± S.E. of four experiments and analysed by Students *t*-test. * $P < 0.05$, ** $P < 0.01$ as compared to control. (A) Phospho-MAP kinase; (B) MAP kinase; (C) ratio of phospho-MAP kinase to MAP kinase. (1) Control; (2) ethanol; (3) MK-801; (4) flurazepam.

4. Discussion

In this study, we demonstrate that ethanol inhibits the phosphorylation of MAP kinase in mouse cerebral cortex. Our data show that ethanol at various concentrations (1.5, 2.5, 3.5 g/kg body weight) decreased the phosphorylation of MAP kinase in vivo. The ethanol-mediated inhibition of MAP kinase phosphorylation has been previously shown in cultured cortical neurons (Kalluri and Ticku, 2000). Several other studies have shown that ethanol can decrease (Davis et al., 1999; Arabi et al., 1999) or increase (Weng et al., 2000; Roivainen et al., 1995) the activation of MAP kinase. A recent report has demonstrated that ethanol did not alter the phosphorylation of ERK in neuroblastoma cell line (McAlhany et al., 2000). Several biochemical parameters that influence MAP kinase phosphorylation are affected by acute ethanol administration in brain tissue. Hence an alteration in the phospho-MAP kinase levels is possible following ethanol intoxication. Time course study has revealed that ethanol decreased the phosphorylation of MAP kinase from 5 to 60 min and reverted back to control levels by 90 min following ethanol administration, suggesting the reversibility of the reaction.

The phosphorylated MAP kinase in the cytosol can either activate other proteins and/or can enter the nucleus to initiate changes in gene expression. Since ethanol decreased the phospho-MAP kinase staining, we further investigated the subcellular distribution of this enzyme. Results indicate that ethanol decreased the phosphorylation of MAP kinase in both cytosol and nucleus, but did not influence the translocation of the enzyme into the nucleus. In contrast, a recent study has reported the translocation of MAP kinase into the nucleus following the treatment of BNLCL2 liver cells with ethanol for 24 h (Reddy and Shukla, 2000). The discrepancy between their data and our observation could be due to the tissue specific difference(s). The brain contains a variety of receptors like NMDA, GABA and ion channels that can potentially alter the phosphorylation of MAP kinase. Studies showing the activation of MAP kinase by Ca^{2+} via NMDA receptors or voltage-dependent Ca^{2+} channels (VDCC) have been very well documented (Orban et al., 1999; Rosen et al., 1994; Xia et al., 1996). Electrophysiological evidence has suggested the involvement of VDCC and NMDA receptors in two separate forms of LTP in vivo (Morgan and Teyler, 1999). Since ethanol acutely inhibits both voltage gated Ca^{2+} channels and NMDA receptor-associated Ca^{2+} influx, and that Ca^{2+} entry into cells activates MAP kinase, it would be prudent to assume that a decrease in the Ca^{2+} concentration might have decreased the phosphorylation of MAP kinase. Likewise, activation of Ca^{2+} calmodulin-dependent kinase by extracellular Ca^{2+} was also reported in hippocampal neurons (Scholz and Palfrey, 1998). An earlier study has demonstrated a positive modulatory role for Ca^{2+} calmodulin-dependent kinase in the activation of MAP kinase via Ras pathway (Chen et al., 1998b). In view of the fact that acute ethanol inhibits NMDA receptors and potentiates GABA receptor function, we further studied the effect of blocking NMDA receptors or activating GABA receptor on MAP kinase phosphorylation. Intraperitoneal administration of MK-801 or flurazepam decreased the phospho-MAP kinase content following 10 min of their injection. It is not known, however, whether the inhibition of MAP kinase by ethanol is due to its action on NMDA receptors or GABA receptors.

In conclusion, the present study demonstrates that ethanol, at relevant concentrations, inhibits the phosphorylation of MAP kinase in mouse cerebral cortex in vivo. The inhibitory effect of ethanol on phospho-MAP kinase staining is observed in all of the age groups studied (16 days through 4 months). Similarly, MK-801 and flurazepam also decreased the phosphorylation of MAP kinase, suggesting that ethanol may be modulating the MAP kinase phosphorylation by interfering with either NMDA receptors or GABA receptors. Further studies are required to elucidate the exact mechanism involved in the process. Changes in MAP kinase phosphorylation may reflect one of the signaling pathways affected by ethanol and may be relevant in the pathophysiology of alcohol related brain damage.

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References

Arabi, S., Garcia, I., Bauer, J.G., Maier, V.R., 1999. Alcohol (ethanol) inhibits IL-8 and TNF: role of the p38 pathway. J. Immunol. 162, 7441–7445.

Chen, J., Ishac, J.N.E., Dent, P., Kunos, G., Gao, B., 1998a. Effects of ethanol on mitogen activated protein kinase and stress-activated protein kinase cascades in normal and renerating liver. Biochem. J. 334, 669–676.

Chen, H.-J., Rojas-Soto, M., Oguni, A., Kennedy, M.B., 1998b. A synaptic Ras-GTPase activating protein (p135 Syn GAP) inhibited by CaM kinase II. Neuron 20, 895–904.

Davis, M.I., Szarowski, D., Turner, J.N., Morriesett, R.A., Shain, W., 1999. In vivo activation and in situ BDNF-stimulated nuclear translocation of mitogen-activated/extracellular signal-regulated protein kinase is inhibited by ethanol in the developing rat hippocampus. Neurosci. Lett. 272 (2), 95–98.

Hendrickson, J.R., Cahill, A.P., McKillop, H.I., Sitzmann, V.J., Redmond, M.E., 1998. Ethanol inhibits mitogen-activated protein kinase activity and growth of vascular smooth muscle cells in vitro. Eur. J. Pharmacol. 362, 251–259.

Kalluri, H.S.G., Ticku, M.K., 2000. Effect of ethanol on the phosphorylation of MAP kinase. Soc. Neurosci. Abstr. #149.4, 406.

Kornhauser, J.M., Greenberg, M.E., 1997. A kinase to remember: dual roles for MAP kinase in long-term memory. Neuron 18, 839–842.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lovinger, D.M., White, G., Weight, F.F., 1989. Ethanol inhibits NMDA-activated ion channels in hippocampal neurons. Science 243, 1721–1724.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265–275.

McAlhany Jr., E.R., West, R.J., Miranda, C.R., 2000. Glial–derived neurotrophic factor (GDNF) prevents ethanol-induced apoptosis and JUN kinase phosphorylation. Brain Res., Dev. Brain Res. 119, 209–216.

Mehta, A.K., Ticku, M.K., 1999. An update on $GABA_A$ receptors. Brain Res. Rev. 29, 196–217.

Mhatre, M.C., Ticku, M.K., 1992. Chronic ethanol administration alters $GABA_A$ receptor gene expression. Mol. Pharmacol. 42, 415–422.

Mii, S., Khalil, R.A., Morgan, K.G., Ware, J.A., Kent, K.C., 1996. Mitogen-activated protein kinase and proliferation of human vascular smooth muscle cells. Am. J. Physiol. 270, H142–H150.

Miyakawa, T., Yagi, T., Kitazawa, H., Yasuda, M., Kawai, N., Tsuboi, K., Niki, H., 1997. Fyn kinase as a determinant of ethanol sensitivity: relation to NMDA-receptor function. Science 278, 698–701.

Morgan, S.L., Teyler, T.J., 1999. VDCC and NMDARs underlie two forms of LTP in CA1 hippocampus in vivo. J. Neurophysiol. 82, 736–740.

Orban, C.P., Chapman, F.P., Brambilla, R., 1999. Is Ras-MAPK signaling pathway necessary for long term memory formation? Trends Neurosci. 22, 38–44.

Reddy, M.A., Shukla, S.D., 2000. Nuclear activation and translocation of mitogen-activated protein kinase by ethanol in embryonic liver cells. Biochim. Biophys. Acta 1497, 271–278.

Roivainen, R., Hundle, B., Messing, R.O., 1995. Ethanol enhances growth factor activation of mitogen-activated protein kinases by a protein kinase C-dependent mechanism. Proc. Natl. Acad. Sci. 92, 1891–1895.

Rosen, B.L., Ginty, D.D., Weber, J.M., Greenberg, E.M., 1994. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 12, 1207–1221.

Sala, C., Rudolph-Correia, S., Sheng, M., 2000. Developmentally regulated NMDA receptor-dependent dephosphorylation of cAMP response element-binding protein (CREB) in hippocampal neurons. J Neurosci. 20 (10), 3529–3536.

Sale, E.M., Atkinson, P.G., Sale, G.J., 1995. Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J. 14 (4), 674–684.

Scholz, K.W., Palfrey, C.H., 1998. Activation of calcium/calmodulin-dependent protein kinase II by extra cellular calcium in cultured hippocampal pyramidal neurons. J. Neurochem. 71, 580–591.

Skattebol, A., Rabin, R.A., 1987. Effect of ethanol on $45Ca^{2+}$ uptake in synaptosomes and in PC12 cells. Biochem. Pharmacol. 36, 2227–2229.

Towbin, H.T., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. 76, 4350–4354.

Weng, Yu.-I., Shivendra, D., Shukla, S.D., 2000. Ethanol alters angiotensin II stimulated mitogen activated protein kinase in hepatocytes: agonist selectivity and ethanol metabolic independence. Eur. J. Pharmacol. 398, 323–331.

Xia, Z., Dudek, H., Miranti, K.C., Greenberg, M.E., 1996. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J. Neurosci. 16 (17), 5425–5436.