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# Effect of short-term ethanol on the proliferative response of Swiss 3T3 cells to mitogenic growth factors

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Abbreviations: DMEM, Dulbecco's modified Eagles medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; JNK, c-Jun $NH_2$-terminal protein kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PDGF, platelet-derived growth factor

## Abstract

**Both adaptive and deleterious responses of cells to ethanol are likely triggered by short-term interactions of the cells with ethanol. Many studies have demonstrated the direct effect of ethanol on growth factor-stimulated cell proliferation. Using Swiss 3T3 cells whose growth was inhibited by ethanol in a concentration-dependent manner, we further investigated the molecular mechanisms of acute ethanol treatment by examining its effect on EGF- and PDGF-mediated cellular signaling systems for the mitogenic function. Tyrosine autophosphorylation of the growth factor receptors was partially prevented by ethanol in intact cells. When ethanol was included before or after EGF stimulation, no effect on the receptor signaling was observed. Here we also report that ethanol inhibits activation of ERK induced by both EGF and PDGF. EGF-induced JNK activation was reduced but PDGF-induced rapid JNK activation was delayed by the addition of ethanol. The balance between its inhibitory and stimulatory effect on the signaling molecules might determine the rate of cell growth.**

**Keywords:** Swiss 3T3 fibroblast, ethanol, EGF, PDGF, ERK, JNK/SAPK

## Introduction

Tissue injury by either acute or chronic exposure to ethanol is due to several factors including accumulation of acetaldehyde and modified proteins, alterations in cellular redox state, microsomal activation of toxins, deranged mitochondrial function, and the enhancement of lymphocyte cytotoxicity (Devi *et al*., 1993; Lieber, 1994; Arnon *et al*., 1995). In addition, ethanol also interferes with the proliferation of several diverse cell lines *in vitro*, including astrocytes, human T cells, and primary rat hepatocytes. The anti-proliferative effects of ethanol may contribute to the pathogenesis and progression of alcoholic diseases. Alcoholic liver disease is a direct result of impaired hepatic regenerative activity due to the inhibition of growth factor-stimulated DNA synthesis (Carter and Wands, 1985). A disruption of neuronal cell proliferation by ethanol has been shown to contribute to fetal alcohol syndrome (Guizzetti and Costa, 1996; Luo and Miller, 1997b, 1999).

Many studies have attempted to define the cellular and molecular mechanisms in the anti-proliferative actions of ethanol. The blockage of growth factor-mediated cell proliferation by ethanol in several systems elicits the hypothesis that growth factor-induced signaling processes are targets of ethanol toxicity. Included are adenylate cyclase (Hoek *et al*., 1992; Williams and Kelly, 1993), protein kinase C (Pandy, 1996), protein tyrosine kinases (Miyakawa *et al*., 1997, Resnicoff *et al*., 1993, Thurston and Shukla, 1992), MAPKs (Roivainen *et al*., 1995; Reddy and Shukla, 1996; Chen *et al*., 1998; Tombes *et al*., 1998; Reddy and Shukla, 2000), phospholipase C and D (Higashi and Hoek, 1991; Hoek *et al*., 1992; Thurston and Shukla, 1992; Saso *et al*., 1997; Zhang and Farrell, 1999), transcription factors such as NF-κB (Zeldin *et al*, 1996) and STAT3 (Chen *et al*., 1997).

However, ethanol is not a universal inhibitor of cell growth. The effect of ethanol on those signaling molecules varies according to the tissues and cell lines. Ethanol also showed different responses depending on the type of growth factors. The differing expression of growth factor receptors and proliferative responses to each growth factor might explain the differential sensitivity of cells to ethanol (Luo and Miller, 1997a). The effect of ethanol is also different depending on whether its action is acute or chronic, and the concentration of both ethanol itself and the growth factor(s). In an effort to define the site(s) of the ethanol action, we further investigated the mechanism by which acute ethanol modulates growth factor responses.

Using Swiss 3T3 cells whose growth is inhibited by ethanol dose-dependently, we further studied the effect of ethanol on DNA synthesis and a cellular signal transduction system activated by two major mitogens, epidermal growth factor (EGF)- and platelet-derived growth factor (PDGF) by examining tyrosine phosphorylation of cellular proteins including the receptors for both PDGF and EGF which elicit many cellular responses via activation of intrinsic tyrosine kinase. To investigate the mechanism by which ethanol modulates growth factor responses, we also examined the activity of mitogen-activated protein kinases (MAPKs), including ERK and c-Jun $NH_2$-terminal protein kinases (JNKs), also known as stress-activated protein kinases (SAPK), which play a pivotal role in various biological events including cell proliferation and differentiation.

## Materials and Methods

### Materials

An ECL detection kit, [$^3$H]thymidine, and [$\gamma$-$^{32}$P]ATP were purchased from Amersham Life Science (Buckinghamshire, England); phenyl methylsulfonyl fluoride (PMSF), aprotinin, Dulbecco's modified Eagle's medium (DMEM), dithiothreitol (DTT), protein A agarose beads, myelin basic protein (MBP) from Sigma (St.Louis, MO, USA); paraformaldehyde from Merck (Darmstadt, Germany); benzamidine hydrochloride from Aldrich (Milwaukee, WI, USA); fetal bovine serum and antibiotics (penicillin and streptomycin) from Gibco/BRL Life Technologies, Inc. (Gaithersburg, MD, USA); Immobilon-P from Millipore (Bedford, MA, USA); horseradish peroxidase-conjugated anti-rabbit IgGs from Vector Laboratory (Burlingame, CA, USA); a BCA protein assay kit from Pierce (Rockford, IL, USA); polyclonal antibodies against phosphotyrosine, and c-Jun (1-169) GST-glutathione agarose conjugate from Upstate Biotechnology. EGF was from Calbiochem (San Diego, CA, USA); PDGF from Bohringer Mannheim.

### Cell Cultures

Swiss 3T3 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 37°C in a humidified, $CO_2$-controlled (5%) incubator. For all experiments, cells were grown on 10-cm culture dishes to subconfluency (about 80-90%).

### MTT Assay

Swiss 3T3 cells ($3 \times 10^5$ cells/ml) were grown on wells in a 24 well plate and treated with ethanol as described in the figure legend. Cells were washed with phosphate-buffered saline (PBS) once and one milliliter of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) solution (2 mg/ml PBS) was added to each well. The plates were incubated at 37°C for 4 h. After MTT solution was aspirated and cells were PBS-washed once, one ml of DMSO was added to dissolve the blue insoluble MTT formazan produced by mitochondrial succinate dehydrogenase. The optical density was measured at 570 nm using a spectrophotometer (Kontron Uvikon 930). The percentage of viable cells was calculated as the relative ratio of optical densities.

### DNA Synthesis

Swiss 3T3 cells grown on 6 well plates were treated with mitogens in the presence or absence of ethanol for 16 h and labeled with 1 μCi/ml of [$^3$H]thymidine for 4 h. Cells were then washed with ice-cold PBS twice and fixed with ice cold 10% TCA. After incubation for 30 min on ice, cells were washed once more with 10% TCA and diethylether/ethanol mixture (1 : 2). Plates were air-dried and 0.75 ml of 0.5 M NaOH to each well was added to dissolve DNA. One half millilitter of aliquots was neutralized by an aliquot of 6N HCl and the radioactivity was counted in 5 ml of Ready Safe scintillation cocktail.

### Western Blot Analysis

Cells were lysed in 2 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 5 mM benzamidine, and 1% Nonidet P-40) for 30 min on a rocker at 4°C. The lysates were spun at 13,000 rpm for 15 min in an Eppendorf centrifuge and the protein concentration of the supernatant was determined using the BCA protein assay kit as described in the protocol supplied by the manufacturer. The same amount of soluble protein from each lysate was analyzed by SDS-PAGE (12%). Proteins were then transferred electrophoretically onto Immobilon P membranes. The blots were blocked by incubating with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 1 hr at room temperature. The blots were then incubated with polyclonal anti-phosphotyrosine antibodies (1 : 1000) in the blocking solution overnight. The blots were washed three times with TTBS and further incubated for 30 min with horseradish peroxidase-conjugated anti-rabbit IgGs (1 : 5000). After washing with TTBS, the blots were visualized using an ECL detection system as described in the manufacturers protocol.

### In Gel ERK Assay

Cells were treated as described in the figure legend and lysed with 2 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 5 mM benzamidine, 1% Triton X-100, 5% glycerol for 30 min. The ERK activity was assayed by a modification of in-gel

kinase assay described by Hibi *et al.* (1993). The clear lysates were prepared by spinning in an Eppendorf centrifuge at 14,000 rpm for 15 min. The lysates boiled in equal volume of 2x SDS sample buffer for 5 min and separated on a 10% polyacrylamide gel containing 0.25 mg/ml MBP. ERKs on the gel was renatured by incubating the gel sequencially with buffer A (20% 2-propanol in 50 mM Tris-HCl, pH 8.0), B (5 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0), C (286.6 g/500 ml guanidine-HCl, 5 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0), and D (0.05% Tween 20 and 5 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0) on a rocker at RT. Each buffer was included for 30 min twice. The gel was finally preincubated with 25 mM kinase reaction buffer containing 40 mM HEPES/KOH, pH 8.0, 100 μM EGTA, 5 mM $MgCl_2$, and 2 mM DTT for 30 min at RT. The kinase reaction in the gel was performed by incubation with hot (50 μCi) and cold (10 mM) ATP for 1 h at RT and terminated by washing them with a washing buffer (5% TCA, 1% Na-pyrophosphate). The gel was washed until the radioactivity was reduced down to the background level. The phosphorylated MBP on the dried gel was visualized by autoradiography.

### Measurement of JNK Activity

The JNK proteins are activated by dual phosphorylation at a Thr-Pro-Tyr motif. We examined the increase of phosphorylated JNK as an indication of JNK activation by SDS-PAGE and Western blot analysis against anti phospho-JNK (G-7). The JNK activity was also assayed by a modification of *in vitro* c-Jun kinase assay on agarose beads described by Hibi *et al.* (1993). The cells were lysed in 2 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 5 mM benzamidine, 1% Triton X-100, 10% glycerol). Cell lysates were incubated with c-Jun (1-169) GST-agarose conjugate (2 μg c-Jun protein in 20 l suspension) at 4°C for 1 hour and the beads were washed twice with a lysis buffer and twice with a basic buffer for kinase assay containing 20 mM Tris-HCl, pH 7.5, 2 mM DTT, and 20 mM $MgCl_2$. The beads were incubated with hot and cold ATP mix (25 μM ATP and 5 μCi $\gamma$-$^{32}$P ATP) in 50 μl of basic kinase assay buffer for 10 min at 30°C. Adding 2× SDS sample buffer and subsequent boiling in a water bath stopped the reaction. The phosphorylated c-Jun was separated on 10% polyacrylamide gel by SDS-PAGE and the dried gel was analyzed by autoradiography.

## Results

### Ethanol inhibits the growth of Swiss 3T3 fibroblasts

In order to study the molecular mechanisms of the ethanol effect on cell growth, a well characterized cell line, Swiss 3T3 fibroblasts was selected. The cells have been extensively studied for the cellular responses against serum containing various mitogens. The cells are quiescent by serum starvation and reinitiate to enter the cell cycle by adding the serum or individual mitogens including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). The potency of ethanol effect on cell growth is known to vary according to tissues and cells. The initial effect of ethanol on cell growth was monitored by measuring the amount of viable cells by MTT assay or counting the number of cells. After 3 days' incubation of cells with various concentration of ethanol, viable cells were reduced dose-dependently in ethanol-treated cells (Figure 1A). There was no visible cytotoxic effect to induce cell death at the concentration range of 50-300 mM ethanol, and most of following study was done within this range of ethanol concentration.

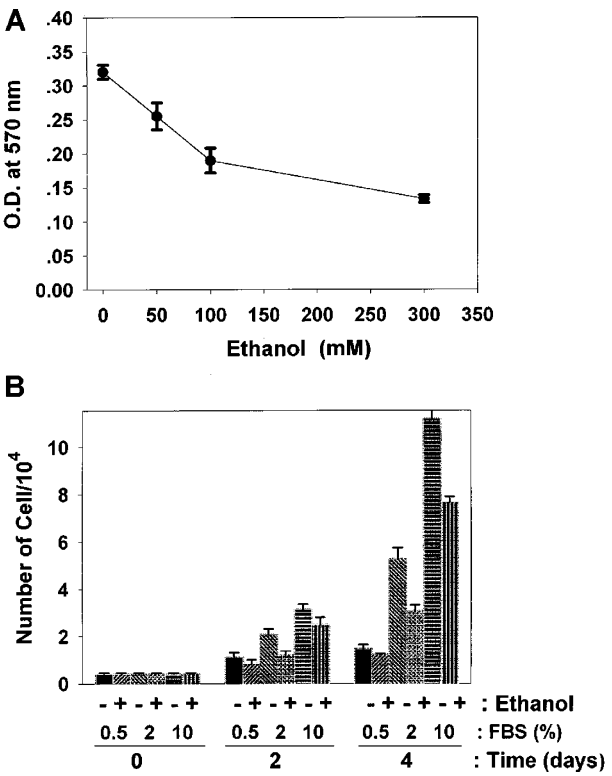


**Figure 1.** Effect of ethanol on the growth of Swiss 3T3 fibroblasts. **A**. Swiss 3T3 fibroblasts were grown on 24-well plates in the culture medium containing 10% FBS for 1 day and the medium was changed to the culture medium with various concentration of ethanol. After 3 days' incubation, the viability of cells was examined by MTT assay. **B**. The culture medium for the cells grown on 24 well plates were treated various amount of FBS (0.5, 2, or 10%) in the absence (–) or presence (+) of 100 mM ethanol. The number of cells was counted at 2 and 4 days after ethanol treatment using a cell counting chamber. Each data represents a mean and standard error. The experiment repeated twice and the results were similar.

The ethanol effect upon the various levels of FBS was examined by adding 100 mM ethanol to cells growing in the culture medium containing 0.5, 2, or 10% of FBS and counted the number of cells after 2 and 4 days' incubation (Figure 1B). Cells did not grow well in the culture medium containing 0.5% FBS. In this case, the ethanol effect was not significant. The cell growth increased by the addition of 2% FBS, but ethanol reduced it to 40-50% of the level observed in the ethanol-untreated control. When the same amount of ethanol was added to the culture medium including10% FBS, a lower rate of reduction, that is, only 20-30% reduction of cell growth was observed. Cells seem to overcome the inhibitory effect of ethanol by the higher concentration of serum. Our data demonstrates that ethanol exerts a differential effect on serum-induced cell growth depending on the concentration of serum.

### Ethanol inhibits growth factor-induced DNA synthesis

EGF and PDGF are selected as representitive mitogens for the Swiss 3T3 fibroblasts and the ethanol effect was examined by DNA synthesis post growth factor treatment of cells. DNA synthesis was examined by measuring the [$^3$H]thymidine incorporation to the serum-starved, quiescent cells at 16h after the addition of the growth factors. The incorporation of [$^3$H]thymidine into DNA increased with increasing concentration of EGF (Figure 2A) or PDGF (Figure 2B). Maximal stimulation of [$^3$H]thymidine incorporation occurred in the presence of 50 nM EGF or 25 ng/ml of PDGF. At the higher concentration of EGF than 50 nM or PDGF than 25 ng/ml, decreased incorporation of [$^3$H]thymidine was seen. Decreasing [$^3$H]thymidine incorporation at the higher concentration of EGF was much more dramatic. The inhibitory effect of high concentration of EGF was also shown in HF cells (Carpenter and Cohen, 1976) and in A431 cells (Kawamoto *et al*., 1983). The addition of high concentration of EGF induces sustained expression of a CDK inhibitor p21waf1/cip1, which might be an important mediator of EGF-induced G1 arrest and growth inhibition (Fan *et al*., 1995). As shown in Figure 2A, EGF-induced DNA synthesis was dramatically reduced in the presence of ethanol. There was a reduction of net increase in [$^3$H]thymidine incorporation: 68% at 1 nM, 63% at 10 nM, 87% at 50 nM, and 77% at 100 nM of EGF. Ethanol exerted less effect on PDGF-induced DNA synthesis (Figure 2B). About 20-30% reduction of net increase was observed.

### Ethanol inhibits growth factor-induced protein tyrosine phosphorylation

EGF- and PDGF-activated signal transduction pathways upon ethanol treatment was investigated. Both EGF and PDGF elicit many cellular responses via activation of intrinsic tyrosine kinase in their corresponding receptors.

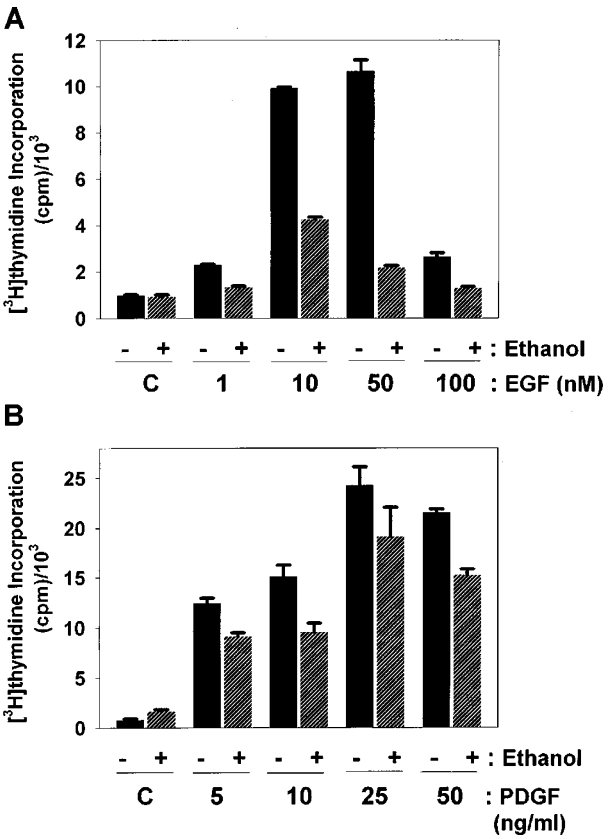


**Figure 2.** Effect of ethanol on EGF- and PDGF-induced DNA synthesis in Swiss 3T3 fibroblasts. Swiss 3T3 cells grown for 2 days in 6 well plates were serum-starved for 2 days by incubation with serum free medium. Cells were stimulated by adding various concentrations of EGF (**A**) or PDGF (**B**) for 16 hours. One half μCi/ml of [$^3$H]thymidine was then added to label the cells for 4 h. DNA was extracted and the radioactivity incorporated into DNA was counted as described in Materials and Methods. The data represents an average of 3 values of count as cpm.

Subsequent tyrosine phosphorylation of the receptors and many other cellular proteins induces mitogenic signals. In order to test if ethanol affects the initial event, we examined its effect on tyrosine phosphorylation of cellular proteins including the receptors. Tyrosine phosphorylated proteins were visualized by SDS-PAGE and Western blot analysis against polyclonal anti-phosphotyrosine antibodies. The presence of tyrosine-phosphorylated proteins of 178-185, 120, 100, 85, 53 kD was detected in EGF- or PDGF-stimulated cells. The tyrosine-phosphorylated 178 and 185 kD proteins might be the activated EGF and PDGF receptor, respectively. Ethanol diminished the level of tyrosine phosphorylation in cells treated with 1-10 nM of EGF (Figure 3A) or 1-10 ng/ml of PDGF (Figure 3B). When the higher concentrations of EGF (100 nM) or PDGF (50 ng/ml) were added to the medium, the ethanol effect was reduced.

To characterize further whether ethanol exerts its effect by modulating the receptor binding of the growth factors or by intracellular metabolites, the cells were preincubat-

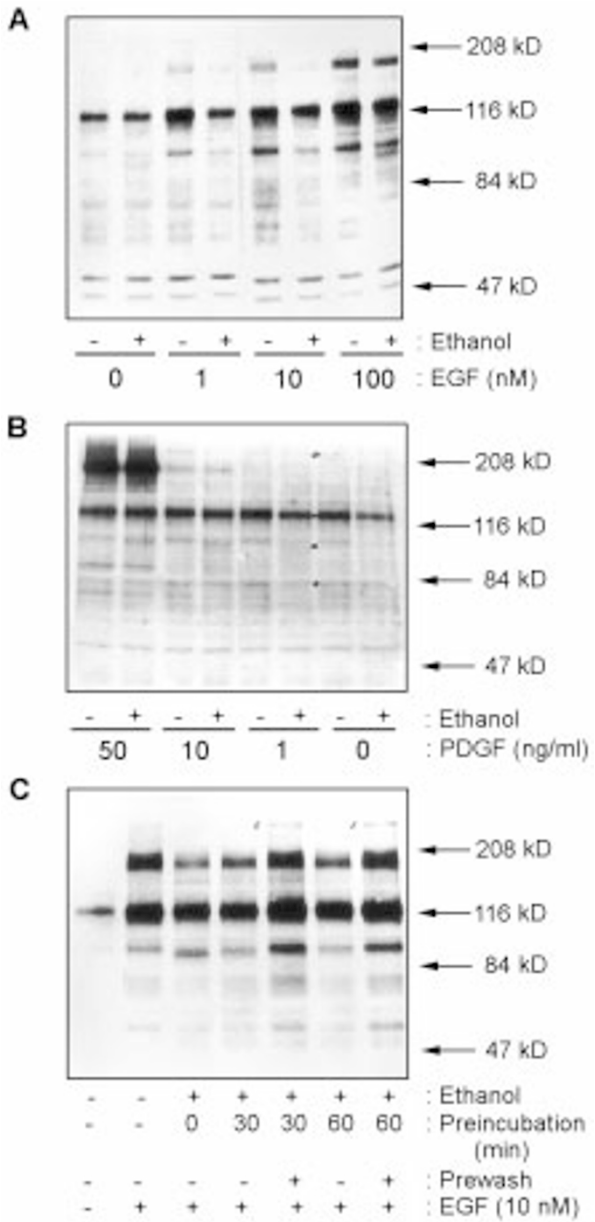


**Figure 3.** Effect of ethanol on EGF- and PDGF-induced activation of protein tyrosine phosphorylation. Cells were serum-starved for 1 day and treated with various density of EGF (**A**) or PDGF (**B**) in the absence (–) or in the presence (+) of 100 mM ethanol. The quescent cells were preincubated with 100 mM ethanol for 0-60 min (**C**). After then, cells were PBS-washed to remove extracellular ethanol (prewash +) and the cells were treated with 10 nM of EGF. Cells were lysed at 5 min after the treatment with the growth factor and the tyrosine phosphorylated proteins were visualized by SDS-PAGE and Western blot analysis using polyclonal anti-phosphotyrosine antibodies.

ed with ethanol for 30-60 min and then ethanol was washed out. EGF treatment for 5 min induced cellular tyrosine phosphorylation in ethanol-pretreated and washed cells similar to that in the untreated cells (Figure 3C). Once ethanol was removed, its inhibitory effect disappeared. Such result indicates that ethanol should be

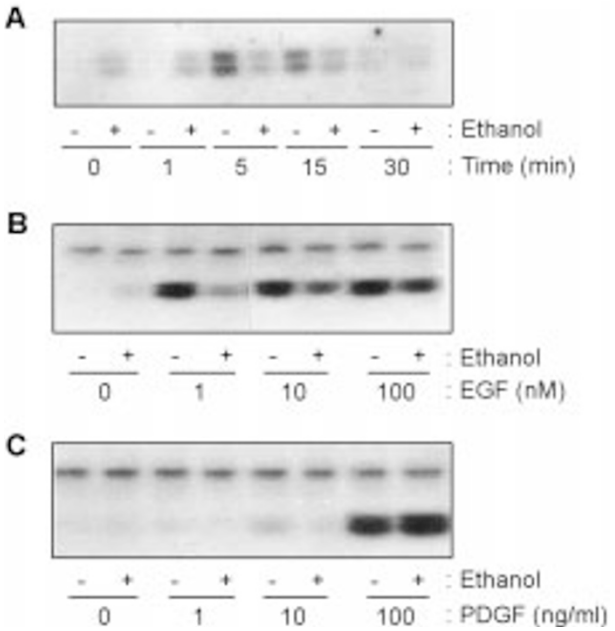


**Figure 4.** Effect of ethanol on the growth factor-induced ERK activation. **A**. Cells were serum-starved for 1 day and lysed at the indicated time after the treatment with 10 nM EGF in the absence (–) or presence (+) of 100 mM ethanol. **B**. The serum-starved cells were treated with various concentration of EGF in the absence or presence of 100 mM ethanol and lysed after 5 min. **C**. The serum-starved cells were treated with various concentration of PDGF in the absence or presence of 100 mM ethanol and lysed after 5 min. ERKs activation was measured in the cell lysates by in-gel kinase assay as described in "Materials and Methods".

present in the medium when cells were treated with the growth factors in order to affect the cellular signaling events.

### Ethanol modulates the growth factor-induced MAPK activity

Mitogen-activated protein kinases (MAPKs) are involved in the integration of multiple signaling pathways leading to cellular responses. EGF and PDGF stimulated the two MAPKs, ERK and JNK, via ras signaling system. To investigate whether acute exposure to ethanol alters the MAPK activation, ERK activity were measured by in-gel ERK kinase assay and JNK activity by a solid bead assay using c-Jun GST-glutathione agarose conjugate. Ten nM of EGF stimulated ERK with a peak at 5 min (Figure 4A). After 15 min, the ERK activity decreased slowly to the basal level during 30 min. In the presence of 100-300 mM ethanol, basal level of ERK activity increased but ERK activation by EGF was completely blocked during the experimental period. In contrast to the results observed in the concentration range of 1-10 nM EGF, the addition of 100 nM EGF relieved the ethanol effect (Figure 4B). Similar phenomenon was observed for the PDGF-induced ERK activation (Figure 4C).

EGF-stimulated JNK activity peaked at 5-10 min and returned to the basal level after 10 min slowly. In the

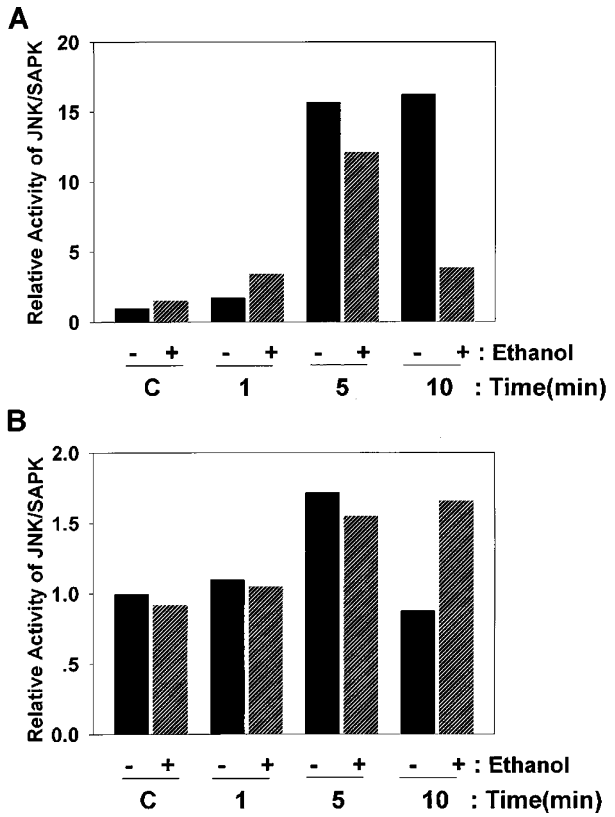


**Figure 5.** The growth factor-induced activation of JNK in ethanol-treated Swiss 3T3 fibroblasts. Cells were serum-starved for 1 day and lysed at 1, 5, and 10 min after the treatment with 10 nM EGF (**A**) or 10 ng/ml of PDGF (**B**) in the absence or presence of 100 mM ethanol. The activity of SAPK/JNK was determined using *in vitro* bead assay as described in "Materials and Methods". The phosphorylated c-Jun protein was separated by SDS-PAGE (8%) and the dried gel was analyzed by autoradiography.

presence of ethanol, the EGF-stimulated JNK activity was reduced slightly at 5 min and returned to the basal level earlier than the control (Figure 5A). PDGF-induced JNK activation was transient with peak at 5 min. At 10 min, the activity returned to the basal level. Interestingly, there was a delayed increase in PDGF-induced JNK activity in the presence of ethanol (Figure 5B). The result tends to suggest that the signaling pathway to activate JNK by EGF and PDGF would be different.

## Discussion

Many studies have demonstrated the direct effect of ethanol on growth factor-stimulated cell proliferation. Ethanol interfered the proliferation of IGF-1-stimulated Balb/c 3T3 cells (Resnicoff *et al.*, 1993) and rat glioblastoma cells (Resnicoff *et al.*, 1994), muscarinic receptor-stimulated glial cells (Guizzetti and Costa, 1996), bFGF- and PDGF-induced neuroblastoma cells (Luo and Miller, 1997b), and PDGF-stimulated astrocyte (Luo and Miller, 1999). However, ethanol does not always modulate the action of all growth factors. Some growth factors are targets of ethanol toxicity but the others are not (Higashi and Hoek, 1991; Luo and Miller, 1997a). Pretreatment of the cells with sublethal concentrations of ethanol markedly inhibited a subsequent stimulation of phospholipase C by vasopressin, angiotensin II, or epidermal growth factor in isolated rat hepatocytes. By contrast, the effects of the alpha 1-adrenergic agonist phenylephrine and of glucagon were not affected by ethanol pretreatment (Higashi and Hoek, 1991). Moreover, ethanol exerts differential effect on the same growth factor depending on the cell type. Although ethanol inhibits the signaling events by EGF in hepatocytes (Higashi and Hoek, 1991; Saso *et al.*, 1997; Zhang and Farrell, 1999), ethanol did not directly interfere with EGF receptor function in mouse BALB/c3T3 cells (Resnicoff *et al.*, 1996). Ethanol inhibited the proliferation of PDGF-stimulated astrocyte, but some studies could not show the inhibitory effect of ethanol on PDGF-induced increases in cell proliferation (Resnicoff *et al.*, 1994; Guizetti and Costa, 1996) and in DNA synthesis (Tomono and Kiss, 1995; DeVito *et al.*, 1997). The discrepancies among those reports might be due to the potency of the growth factor and ethanol on each cell line. In the present study, we provide evidence that ethanol directly affects both cell growth and growth factor-induced cellular signaling in Swiss 3T3 cells. DNA synthesis and protein tyrosine phosphorylation induced by EGF was seriously distorted by ethanol. The effect of ethanol on PDGF-induced response was weak but concentration-response experiment clearly demonstrated the inhibitory effect of ethanol on PDGF-induced cell proliferation, DNA synthesis and ERK activation. Differential susceptibility of cells to ethanol might correlate with their response to mitogenic growth factors (Luo and Miller, 1997a).

Free radical damage to cell membrane receptors may not be an important mechanism of alcohol-induced inhibition of cell proliferation. Previously, experiments with acetaldehyde and with the alcohol dehydrogenase inhibitor 4-methylpyrazole suggested that the inhibitory effect was due to ethanol itself and not to its metabolite acetaldehyde (Guizzetti and Costa, 1996). Alleviation of cellular radical production alone was not sufficient to prevent the abnormality in fetal rat hepatocyte function (Devi *et al.*, 1993). When ethanol was pretreated for 1 hour and removed by PBS-washing, there were no changes in the growth factor-induced signaling pathways. Taken together, it can be concluded that ethanol should be present with growth factors at the same time to exert any effect on the signaling events.

Our data thus showed that the addition of excess amount of growth factors reduced the inhibitory effect of ethanol and is agreeable with the report by Higashi and Hoek (1991) demonstrating that ethanol effects were pronounced at low concentrations of agonists but they were not significant at saturating levels in hepatocytes.

Studies using buccal mucosa and rat gastric mucosa demonstrated the reduction in the EGF receptor binding by ethanol (Wang *et al*., 1992, 1994). Chronic ethanol feeding causes a time-dependent reduction of the EGF binding to the receptor in rat stomach due to the reduction of EGF receptor number, and/or the affinity of the high-affinity binding site (Wang *et al*., 1996, 1997). In hepatocytes isolated from chronic ethanol-fed rats, binding to both high and low affinity states of the hepatic epidermal growth factor receptor was decreased by 40-50% (O'Rourke *et al*., 1997). It might cause a concomitant decrease in the ability of the receptor tyrosine kinase to phosphorylate tyrosine residues. It raises the question of what causes the alteration of the affinity of growth factor to the receptor. There is a possibility that EGF receptor is damaged by ethanol-induced free radical as shown in the case of glucagon receptor (Shaw *et al*., 1995). Otherwise, the membrane environment could be altered to reduce the interaction between the growth factor and receptor. Ethanol induced activation of phospholipases C and D could metabolize the membrane lipids and rapidly produce unusual lipids such as phosphatidylethanol (Alling *et al*., 1984; Gustavsson *et al*., 1991). A lack of mitogenic product of phospholipases including phosphatidic acid might be one of the mechanisms that contributes to the inhibition of cell growth by ethanol (Kotter and Klein, 1999).

Ethanol itself activates some of signaling molecules but it blocks or potentiates their induction by other stimulants such as growth factors and hormones. Ethanol itself activates PLC (Rubin and Hoek, 1988; Rooney *et al*., 1989) whose product, diacylglycerol, in turn activates protein kinase C, which desensitizes agonist-induced activation of PLC (Higashi and Hoek, 1991). Treatment of hepatocytes with 50 mM ethanol elevated the basal activity of p42 ERK (Thombes *et al*., 1998). In this study, we also observed an elevation of basal ERK activity by 10 min incubation with 100 mM ethanol. Ethanol might activate those signaling proteins by a pertussis toxin-sensitive G-protein-dependent mechanism (Reddy and Shukla, 1996). In most cases, ethanol inhibits growth factor-induced receptor tyrosine autophosphorylation (Thurston, Jr. and Shukla, 1992; Resnicoff *et al*., 1993, 1994; Bhavani *et al*., 1995), which subsequently interferes with the activation of key down stream signaling mediators including PLC-g1 (Higashi and Hoek, 1991; Thurston, Jr. and Shukla, 1992; Saso *et al*., 1997; Zhang and Farrell, 1999), IRS-1 (Resnicoff *et al*., 1994; Bhavani *et al*., 1995), phosphatidylinositol-3 kinase (Resnicoff *et al*., 1994), and MAP kinases (Banerjee *et al*., 1998; Thombes *et al*., 1998; Seiler *et al*., 2000), and transcription factors such as c-myc, c-fos, and c-jun (Resnicoff *et al*., 1993). We also observed an inhibition of EGF- or PDGF-stimulated ERK activity.

It is not always true that the adverse effects of ethanol are mediated by inhibition of tyrosyl phosphorylation of some signaling molecules including the growth factor receptor. Although long-term ethanol feeding suppresses EGF-induced receptor autophosphorylation in rat hepatocytes, it shows a differential in the inhibition of downstream signaling processes mediated by PLC, Shc, and Grb2 (Saso *et al*., 1997). In FOCUS hepatocellular carcinoma cells, which overexpress IRS-1, ethanol treatment substantially inhibits IRS-1 and MAP kinase signaling and growth-associated gene expression, but has no effect on Shc phosphorylation, which activates p21ras through an IRS-1 independent pathway (Banerjee *et al*., 1998). Ethanol inhibited PDGF-BB-mediated phosphorylation of PDGFRa, but it had little effect on PDGFRb autophosphorylation. Likewise, ethanol abolished the association of PDGFa to Ras GTPase-activating protein (Ras-GAP), but it did not affect the binding of Ras-GAP to PDGFb. PDGF stimulated the activities of mitogen-activated protein kinase (MAPK) in protein kinase C (PKC) independent and dependent manners. Ethanol inhibited the PKC-independent, acute activation of MAPK; however, it stimulated the PKC-dependent, sustained activation of MAPK (Luo and Miller, 1999). Ethanol enhances NGF- and bFGF-stimulated neurite outgrowth in PC12 cells by stimulating phosphorylation and activation of MAP kinases through a delta or epsilon PKC-regulated pathway (Roivainen *et al*., 1995; Pandy, 1996). In our study, ERK activation within 10 min incubation with PDGF or EGF might implicate the ERK activation via PKC-independent pathway.

We demonstrated that ethanol exerts a potential inhibitory effect on JNK activation by EGF. However, it rather stimulated PDGF-induced JNK activation at 10 min. The differential effect of EGF and PDGF on JNK seems to suggest that the JNK activation pathway would be different in the two growth factors. Subsequent autophosphorylation of the receptors or their phosphorylation by receptor-associated protein kinases has been speculated to induce the activation of MAPK cascade including ERK and JNK (Pomerance *et al*., 1998). It was suggested that the dynamic balance between ERK and JNKs or p38 and the duration of MAPK activation would be important in determining the final cellular fate (Xia *et al*., 1995; Marshall, 1995; Chen *et al*., 1996; Kang *et al*., 1999). Differential effect of ethanol on the growth factor-stimulated ERK and JNK might be another determinant for the final effect of ethanol on cell proliferation.

The effect of ethanol varied according to its concentration and also the incubation time with ethanol. EGF-induced tyrosine phosphorylation of PLC-$\gamma$1, a substrate for EGF receptor tyrosine kinase, in intact A431 cells showed a biphasic pattern (Thurston, Jr. and Shukla, 1992). In the presence of low concentration of ethanol, EGF-stimulated tyrosine kinase was potentiated. However, at higher concentrations of ethanol, a decrease was observed. Acute exposure to ethanol (0-400 mM) for 1 hr had no effect on either basal or serum- and phorbol-

12-myristate-13-acetate (PMA)-stimulated ERK activity in a normal mouse embryonic liver cell line, BNLCL2 (Reddy and Shukla, 1996). Exposure to ethanol for 16-24 h prolongs or potentiates the activation of ERK induced by various agonists in a normal mouse embryonic liver cell line (Reddy and Shukla, 1996) and in primary rat hepatocytes (Chen *et al*., 1998). Exposure to ethanol for 16 h increased the basal JNK activity but there was no change in agonist-induced JNK activation. Whereas chronic ethanol intake for 60 days inhibited the activation of ERK, p38, and JNK induced either by partial hepatectomy or by treatment with various agonists (Zeldin *et al*., 1996; Chen *et al*., 1998). In the present study, we observed that acute ethanol treatment for 5-10 min inhibited growth factor-stimulated tyrosine phosphorylation and ERK activation even in the absence of any preincubation with ethanol. Taken together, these observations seem to provide the molecular mechanism of acute ethanol treatment on cell growth.

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