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Ethanolamine Is a Co-Mitogenic Factor for Proliferation of Primary Hepatocytes

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Abstract Mature adult parenchymal hepatocytes can enter the S phase in the presence of growth factors such as HGF and EGF, but rarely proliferate in culture. We hypothesized that the cell cycle of hepatocytes in culture is restricted before G_2/M phase and we attempted to identify the factor that induces cell cycle progression. We found that the conditioned medium from long-term cultured hepatocytes contained co-mitogenic activity with other growth factors, which was attributed to ethanolamine (Etn). Etn induced not only DNA synthesis but also cell replication of cultured hepatocytes with various other growth factors. Etn and HGF synergistically induced cyclin D_1, A and B expression, however, only cyclin B but not cyclin A formed a complex with Cdc2. In addition, Etn combined with HGF enhanced PKCβII expression and translocated PKCβII to the plasma membrane, and induced filopodia formation, which was inhibited by an antisense oligonucleotide against PKCβII. In addition, blocking the cytoskeleton rearrangement with inhibitors (colchicine, cytochalasin D, or chlerythrine (a specific PKC inhibitor)) inhibited cyclin expression and cell proliferation. Although Etn enhanced the downstream product, cellular phosphatidylethanolamine (PE), PE itself did not show any Etn-like activities on hepatocytes. Taken together, our results indicate that Etn functions as a co-replication factor to promote the cell cycle of mature hepatocytes to G_2/M phase in the presence of growth factors. The activity is thought to be mediated by PKCβII-dependent cyclin B expression. J. Cell. Biochem. 84: 249–263, 2002.

Key words: hepatocytes proliferation; cell cycle; ethanolamine; PKC; cyclin B

The liver is the only metabolic organ to regenerate to the original size after up to 70% of the organ has been surgically removed. The regeneration process is primarily achieved by cell proliferation of mature parenchymal hepatocytes [Grisham, 1962] in the presence of growth factor stimulation [Fausto et al., 1995; Michalopoulos and DeFrances, 1997]. These cells (or a fraction of them) exhibit high capacity for clonal growth, as shown by hepatocyte transplantation experiments in transgenic mouse models [Rhim et al., 1994]. However, despite such a high capacity of mature hepatocytes to proliferate in vivo, these cells rarely proliferate in primary cultures. This limitation has hindered the use of these cells to tissue engineering such as cellular transplantation and gene therapy. Therefore, recently, much attention has been paid on the culture of liver progenitor cells (designated as "oval cells" or "small hepatocytes"), which are known to potentially proliferate and differentiate into both hepatocytes and ductular hepatocytes in vitro [Fausto, 1994; Mitaka, 1998; Mitaka et al., 1999]. However, the population rate of these cells is very low and the proliferation in vitro is inefficiently slow. In addition, the bipotential of these cells for differentiation into two types of hepatocytes is not exclusive since mature hepatocytes could also function as "hepatoblasts" [Block et al., 1996]. Therefore, mature hepatocytes would be a better source for clinical purposes if these cells proliferate in vitro. It is demonstrated that although various growth factors involved in the liver regeneration in vivo also promote cell cycle of primary hepatocytes from G_1 to S phase [Richman et al., 1976; McGowan et al., 1981; Mead and Fausto, 1989; Nakamura et al., 1989], these molecules rarely induce cell division in culture. These facts suggest that the cell cycle of primary hepatocytes in culture is restricted to

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progress to G_2/M phase for some unknown reason. In contrast, several investigators reported recently that modification of the culture medium could induce cell division of primary hepatocytes in culture [Hasegawa et al., 1982; Block et al., 1996; Sato et al., 1999] indicating that the restriction can be, at least in part, overcome. However, there is little information regarding the molecules or mechanisms involved in overcoming the restriction point. We have investigated the type of molecule that promotes cell division of primary hepatocytes in vitro and found that ethanolamine (Etn) functions as a co-cell dividing factor for primary cultured hepatocytes in the presence of growth factors.

Etn is known to enhance DNA synthesis in some cell types [Kano-Sueoka et al., 1983; Kano-Sueoka and King, 1987; Kiss and Crilly, 1996; Kiss et al., 1996, 1997a,b; Malewicz et al., 1998] including hepatocytes [Nelson et al., 1996; Sasaki et al., 1997]. However, the exact mechanism remains to be elucidated. In the present study, we demonstrate that Etn functions not only as a DNA synthesis enhancer (G1 promoter) but also as a co-replicating factor (G_2/M promoter) on primary hepatocytes and that PKCβII-dependent cyclin B plays an essential role in hepatocyte proliferation.

MATERIALS AND METHODS

Reagents

Female C57BL6 (6–12 weeks) and p53-deficient mice used in the present experiments were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Recombinant human HGF was a kind gift from Snow Brand Milk Products Co. (Tokyo, Japan). Antibodies against cyclin A, cyclin B, cyclin D_1, cyclin E, cdc2, PKCα, PKCβII, and PKCζ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse BrdU antibody was purchased from Zymed Laboratories, Inc. (San Francisco, CA). Rhodamin-labeled phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR). Other reagents described in the text were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Parenchymal hepatocytes were prepared as described previously [Morita et al., 1994]. Isolated live parenchymal hepatocytes were suspended in Williams' E medium containing 10% FCS, 20 ng/ml EGF, 10^{-9} M insulin, and antibiotics in RPMI1640 with 10% FCS and were plated at a density of 7.5×10^3 cells/well in flat-bottomed 96-well plates (Sumitomo Bakelite Co., Tokyo) pre-coated with collagen. These cells were cultured in modified HGM medium [Block et al., 1996] (BSA 2 mg/ml, glucose 2 mg/ml, galactose 2 mg/ml, ornithine 0.1 mg/ml, proline 0.03 mg/ml, nicotinamide 0.61 mg/ml, glutamin 0.73 mg/ml, $ZnCl_2$ 0.54 μg/ml, $ZnSO_4 \cdot 7H_2O$ 0.75 μg/ml, $CuSO_4 \cdot 5H_2O$ 0.2 μg/ml, and $MnSO_4$ 0.025 μg/ml in DMEM) in the presence or absence of HGF (10 ng/ml).

Purification and Identification of Etn

One-half liter of the culture supernatant from 7-day cultured rat hepatocytes was harvested and lyophilized. The sample was extracted by ether two times and the aqueous phase was precipitated with acetone. The sample was dissolved in dH_2O, then applied to a Q-sepharose fast flow column (Pharmacia, Uppsala, Sweden) equilibrated with 0.15 M NaCl-20 mM Tris-HCl (pH 8.5). The flow through fractions was collected, lyophilized, and dissolved in 30 ml of dH_2O. The sample was loaded at a rate of 2 ml/min onto a TSK gel ODS 80TM column (Tohso Co.) equilibrated with 20 mM acetate buffer (pH 8.0) and the active fraction was eluted by a gradient of 90% acetonitorile-20 mM acetate buffer (pH 5.6) using ACTA purifier (Pharmacia). Active fractions were collected and concentrated by a vacuum drier to 10 ml, then applied to HiPrep 16/60 Sepharcyl S-200 column equilibrated with H_2O and sequentially to a Superdex Peptide PE column 7.5/300 (Pharmacia). Finally, 400 μl of purified sample was obtained. The sample was then subjected to ^{1}H-NMR analysis and TLC.

Proliferation Assays

Cell proliferation was measured by three methods for distinct criteria. Substantial cell growth was quantified by the crystal violet method as described previously [Ajioka et al., 1999]. Cell numbers in S phase were counted by staining BrdU incorporated cells with FITC-anti BrdU antibody. Finally, the number of cell nuclei was counted in PI (propidium iodide, Sigma) stained cells.

Immunoprecipitation and Western Blotting

Cells (1×10^6) were harvested with lysis buffer A (150 mM NaCl, 1% NP-40, 0.1% sodium

deoxycholate, 0.1% SDS, 1 mM EDTA, 10 μg/ml aprotinin, 100 mM NaF, 0.2 mM Na_3VO_4, and 0.5 mM PMSF) and the samples were centrifuged at 15,000*g* for 30 min to obtain clear lysates. The nuclear fraction was prepared by solubilizing hepatocytes with lysis buffer B (0.1 M citric acid-1% Triton X-100 solution) and centrifugation at 2,700*g* for 2 min. The pellets were dissolved in the SDS–PAGE loading buffer. For isolation of cell membrane and cytosol fraction, cells were lysed in lysis buffer C (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupepsin, 10 μg/ml aprotinin, and 1 mM PMSF). The supernatant representing the cytosol fraction was obtained by centrifugation at 42,000*g* for 30 min. The pellet was then dissolved in lysis buffer B and centrifuged at 15,000*g* for 30 min. The supernatant was collected as the membrane fraction. These solutions were incubated with protein G-Sepharose bearing each indicated antibody for 1 h at 4°C after preincubation with protein G-Sepharose. Sepharose was pelleted by brief centrifugation, then washed with the same buffer three times. The bound protein was extracted by SDS loading buffer and subjected to SDS–PAGE on 12.5% gel. Proteins were then electroblotted onto PVDF membranes (Amersham, IL) in cold transfer buffer for 3 h at 60 V. Filters were incubated with first antibodies for 1 h at room temperature after blocking with 3% skim milk, washed with TBS, and then incubated with peroxidase-labeled second antibodies for 1 h. Bands were detected with an ECL kit (Amersham).

Immunohistochemistry

Cultured hepatocytes on collagen-coated cover glasses were permeabilized with 1% Triton X-100 in cytoskeleton buffer (CB) (10 mM MES (pH 6.1), 150 mM NaCl, 5 mM EDTA, 5 mM $MgCl_2$, and 5 mM glucose) for 30 s and fixed with 1% glutaraldehyde for 10 min. After washing the cells three times, they were treated with $NaBH_4$ for 5 min and then washed with the same buffer and locked with 1% BSA for 5 min. The cells were then incubated with anti-β-tubulin or anti-γ-tubulin for 60 min at room temperature. The first antibodies were visualized with the labeled-second antibodies. For actin or nuclear staining, the fixed cells were incubated with rhodamin-labeled phalloidin or PI. These cells were finally examined by a confocal microscope, TCS-NT (Leica Lasertechnik, Heerbrugg, Switzerland).

PKC Assay

PKC fraction was purified from cultured hepatocytes (3×10^6 cells) as described previously [Adachi et al., 1996]. PKC activities in the fractions were measured by a PepTag Non-Radioactive PKC assay kit (Promega, Madison, WI) based on the instructions provided by the manufacturer.

Antisense Treatment for PKCβII Downregulation

Phosphonotioate antisense oligonucleotides against PKCβII was synthesized according to the sequence employed in Murray et al. [1993] (+31–48) (5′-GCTCTCCTCGCCCTCGCT-3′). The antisense or random oligonucleotides were transfected into primary hepatocytes with the transfection reagent, FuGENE6 (Roche Diagnostics, Mannheim, Germany).

Phospholipids Analysis

Lipid fraction was extracted from stimulated hepatocytes by the method of Bligh and Dyer [1959]. Phospholipids were separated by TLC on silica gel plates in the developing solvent (CH_3Cl:MeOH:28% NH_4OH = 13:7:1). Phospholipids were detected by staining with 0.03% Coomasie Brilliant Blue R250 in 20% methanol and analyzed by NIH image software.

Statistical Analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using one-way ANOVA statistics followed by the Student-Newman-Keul test. A P value of less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Purification and Identification of Etn as a Co-Mitogenic Factor

In contrast to rat hepatocytes, which proliferate in vitro with HGM [Block et al., 1996], mouse hepatocytes did not proliferate in the same medium. However, we found that the supernatant from 7-day cultured mouse hepatocytes contained co-mitogenic activity to induce the proliferation of mouse hepatocytes in the presence of HGF and EGF (Fig. 1A,B).

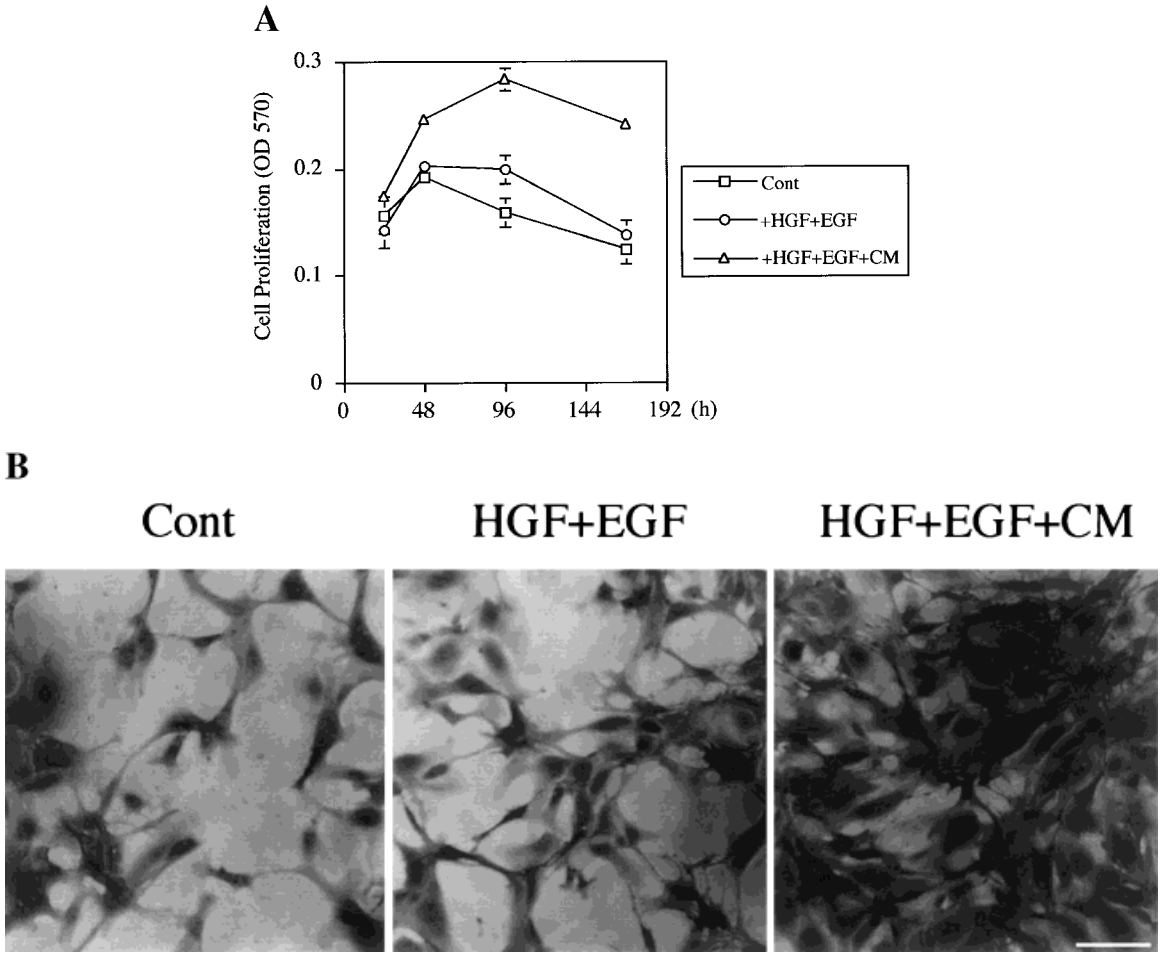


Fig. 1. Conditioned medium of long-term cultured hepatocytes exhibits co-mitogenic activity to primary hepatocytes in the presence of growth factors. Conditioned medium (CM) was harvested from 7-day cultured mouse hepatocytes with HGF (10 ng/ml) and EGF (10 ng/ml), and then equal volume was added to the medium containing freshly isolated mouse hepatocytes. These cells were cultured with HGF (10 ng/ml) and EGF (10 ng/ml) for various time intervals. **A**: Cell growth was evaluated by crystal violet assay. **B**: Microscopic examination of cultured cells after staining with crystal violet. Bar, 100 μm.

The effect was more significant when p53-deficient hepatocytes were stimulated with the conditioned medium and growth factors. On the other hand, p53-deficient hepatocytes themselves did not proliferate without the conditioned medium even in the presence of only growth factors (Fig. 2A,B). Since HGF-stimulated p53-deficient hepatocytes showed abundant DNA synthesis (data not shown), we speculated that the conditioned medium contained co-mitogenic (or cytokinesis-inducing) activity. We then determined the activity in the conditioned medium of 7-day cultured rat hepatocytes. The purification procedures are summarized in Table I. The purified sample was applied to NMR, and based on the spectrum analysis, the molecule was identified as Etn. Etn was also confirmed by TLC analysis (data not shown).

In the next step, we examined the biological activity of Etn as a co-mitogenic factor in the proliferation of primary cultured mouse hepatocytes. Etn synergistically with HGF increased DNA synthesis in primary hepatocytes as previously reported [Nelson et al., 1996]. Interestingly, Etn itself also increased DNA synthesis of hepatocytes (Fig. 3A). The induction of DNA synthesis was transient and reached a peak level at 60 h after stimulation. In addition, costimulation of primary hepatocytes by HGF plus Etn induced a significant increase in mitotic cells at 48 h after stimulation and the nuclear number while HGF or Etn alone did not (Fig. 3B,C). These results also suggest that Etn functions as a co-mitogenic factor for primary hepatocytes. The co-mitogenic activity of Etn on hepatocytes was not only noted with HGF but also with other hepatocyte growth factors

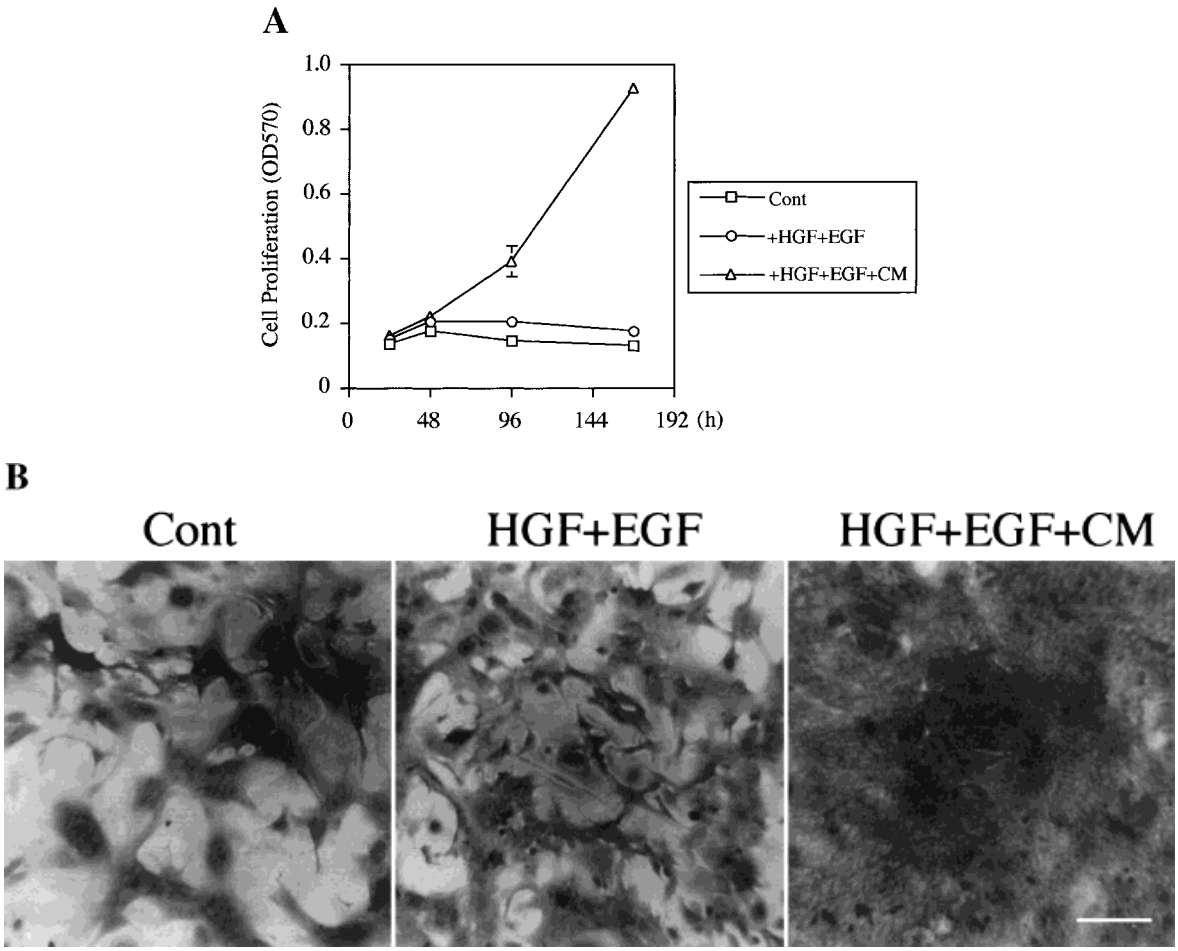


Fig. 2. Marked co-mitogenic activity of the conditioned medium is observed in p53-deficient hepatocytes. Isolated p53-deficient hepatocytes were precultured for 16 h on a collagen-coated plate, and a volume of conditioned medium similar to that of the culture medium was added to the cells in the presence of HGF (10 ng/ml) and EGF (10 ng/ml). The cells were then cultured for various time intervals. **A**: Cell growth was evaluated by crystal violet assay. **B**: Microscopic examination of cultured cells after staining with crystal violet. Bar, 100 μm.

TABLE I. Purification Procedures Used to Identify the Co-Mitogenic (or Cytokinesis-Inducing) Activity of Ethanolamine in the Conditioned Medium of 7-Day Cultured Rat Hepatocytes

	Volume (ml)	Weight (mg)	Arbitrary activity
Conditioned medium	1,500	16,250	1
Acetone-soluble	30	8,250	8.15×10^2
Q-sepharose	30	5,500	1.04×10^3
TSK-GEL ODS	20	2,440	1.71×10^3
Sepharcryl S-200	4	93.8	8.80×10^5
Superdex peptide	0.4	0.89	5.87×10^6

The activity of the original conditional medium to induce the proliferation of primary hepatocytes by two-fold in number was determined as one unit. The relative arbitrary activities of the samples during purification was determined as the reciprocal of dilution number to confer the equivalent to one unit.

including EGF, TGFα, and insulin, but not with TNFα (Fig. 4A), indicating that the specific activity of HGF is irrelevant to the co-mitogenic activity of Etn. In addition, Etn did not exhibit any effect on nonparenchymal cells (Fig. 4B). Therefore, the proliferation reflected the growth of hepatocytes but not that of possible contaminated nonparenchymal cells.

Etn Induces Active Cyclin B but not Cyclin A Expression for Cell Cycle Progression

Cell cycle progression including entering G_2/M phase is regulated by cyclins [King et al., 1994; Nurse, 1994]. Therefore, we examined whether Etn regulates cyclin expression and the type of cyclin, if any. Figure 5A shows that

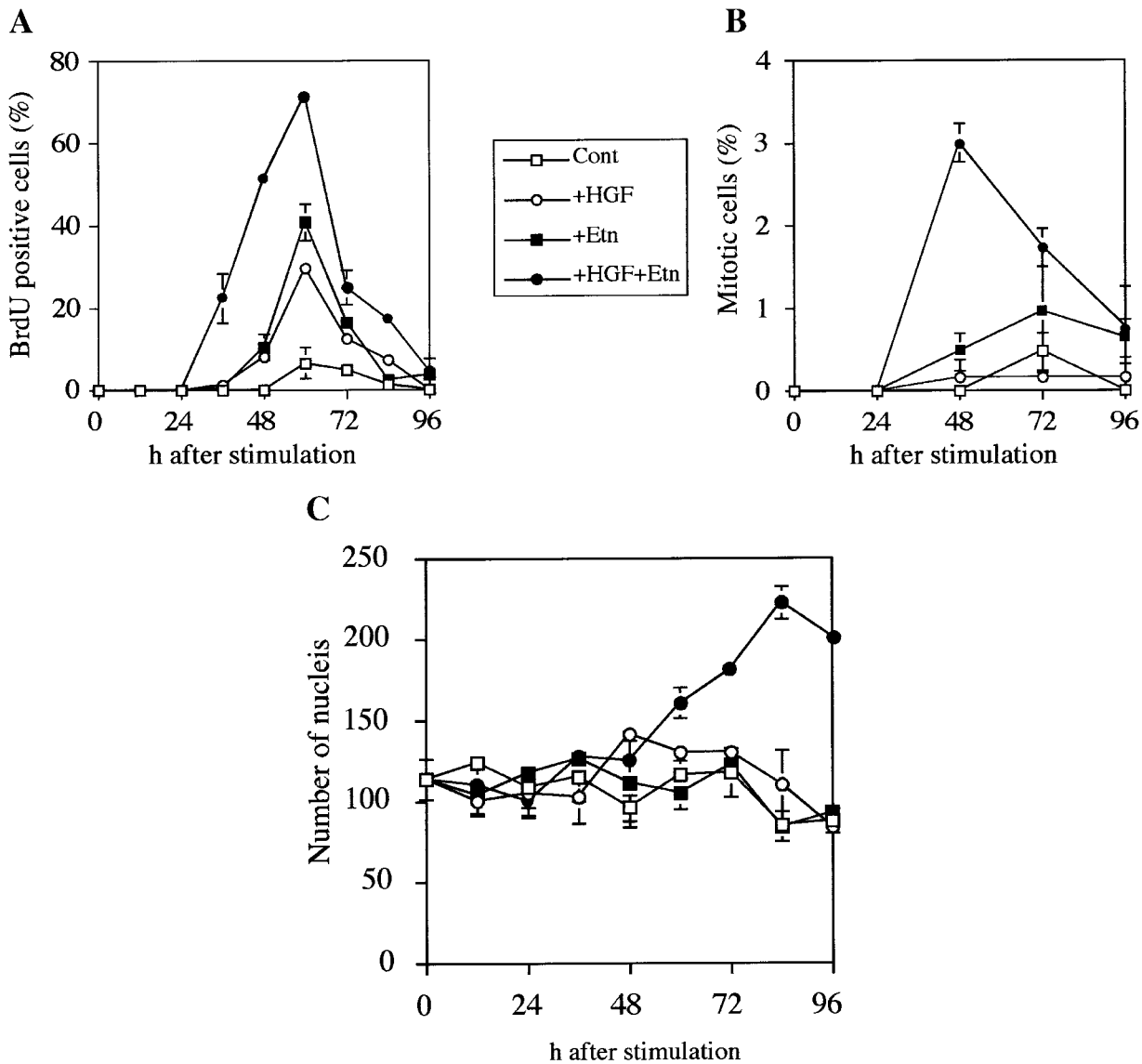


Fig. 3. Ethanolamine (Etn) induces cell division of primary hepatocytes in the presence of HGF. Isolated hepatocytes were incubated with Etn (100 μM) and HGF (10 ng/ml) for various time intervals. **A**: BrdU incorporation was measured to estimate the G_1 to S phase progression. BrdU was added 12 h prior to cell fixation and BrdU incorporated cells were visualized with FITC-anti-BrdU antibody, then counted under a confocal microscope. **B**: Cells were stained with anti-β-tubulin and the mitotic cell number was counted using NIH image analysis. **C**: Cells were fixed and nuclei were stained with PI, and their number was counted in three different fields under a confocal microscope.

Etn significantly enhanced HGF-induced expression of cyclin D_1, A and B at 48 h after stimulation. In contrast, the expression of cyclin E, a G_1 cyclin, appeared spontaneously until 48 h and then disappeared 72 h after stimulation. On the other hand, although Cdc2 expression was increased by HGF plus Etn at 48 h, the expression remained the same at least until 72 h. Since Cdc2 promotes the cell cycle to M-phase by forming a complex with cyclin A or cyclin B [King et al., 1994; Nurse, 1994], we performed immunoprecipitation to check which cyclin is the critical mediator. Our results showed that only cyclin B formed a complex with Cdc2 (Fig. 5B), suggesting that Etn together with HGF induced the transition of hepatocytes to M-phase via cyclin B expression.

Cytoskeleton Rearrangement is Required for HGF Plus Etn-Induced Cyclin B Expression and Cell Division

During the experiments, we found that Etn significantly induced filopodia formation in hepatocytes even in the absence of HGF

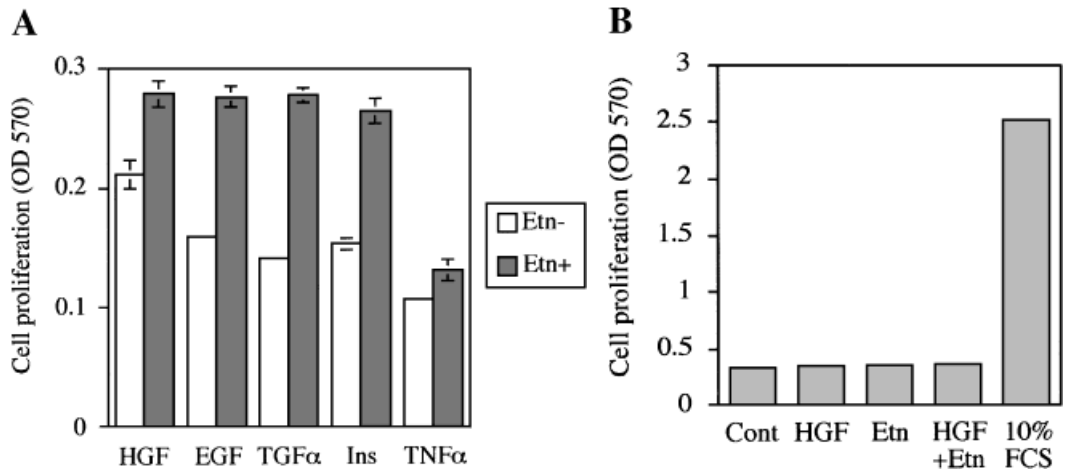


Fig. 4. Ethanolamine specifically induces cell division in hepatocytes incubated with various growth factors but not in nonparenchymal cells. **A**: Isolated hepatocytes were cultured with HGF (10 ng/ml), EGF (10 ng/ml), TGFα (50 ng/ml), insulin (Ins) (10^{-7}M) or TNFα (50 ng/ml) in the presence of Etn (100 μM) for 96 h. Cell proliferation was evaluated by crystal violet assay. **B**: Isolated nonparenchymal cells from p53-deficient mice were cultured with Etn (100 μM) and/or HGF (10 ng/ml) for 96 h and crystal violet assay was performed. These cells were also cultured with 10% FCS as a positive control.

(Fig. 6A,C) while HGF induced stress fiber formation (Fig. 6A,B), demonstrating that Etn regulates particular cytoskeleton rearrangement. Since the cell cycle of adherent cells is controlled by a mechanism involving cytoskeleton rearrangement [Elliott et al., 1992; Mooney et al., 1992; Hansen et al., 1994; Huang et al., 1998; Zhao et al., 1998], we next investigated whether the cytoskeleton rearrangement is involved in Etn-induced cell cycle progression. As shown in Figure 7A, cytochalasin D (an actin inhibitor) and colchicine (a microtubule inhibitor) completely inhibited HGF plus Etn-induced cyclin B expression while the expression of other cyclins was less affected. These reagents simultaneously and completely suppressed the induced cell proliferation (Fig. 7B). These data indicate that both cyclin B expression and cell proliferation signals induced by HGF and Etn were transduced via cytoskeleton rearrangement.

Activated PKCβII Mediates the Signal Transduction of Etn to Filopodia Formation, Cyclin B Expression, and Cell Division of Hepatocytes

Both effects of Etn on cytoskeleton rearrangement and cell cycle progression to M-phase led us to examine the involvement of protein kinase C (PKC) in the signaling pathway since the molecule is thought to be an important mediator of these events [Livneh and Fishman, 1997; Keenan and Kelleher, 1998]. In fact, stimulation of hepatocytes by Etn increased PKC activity (Fig. 8A). Next, we investigated the type of PKC molecule that was activated by Etn. We confirmed that primary mouse hepatocytes express only PKCα (80 kDa), PKCβII (45 kDa), and PKCζ (80, 65 kDa) but not PKCβI, PKCθ, PKCλ as reported for rat hepatocytes [Ducher et al., 1995] (data not shown). Among these, Etn alone or Etn and HGF translocated PKCβII from the cytosol to the membrane but not to the nuclear fraction and enhanced its expression at 48 h after stimulation. In contrast, there were no significant changes in the distribution or expression of PKCα and PKCζ following similar stimulation (Fig. 8B). These data suggest the involvement of PKCβII in Etn-induced effects. Accordingly, we focused on the role of PKCβII in the filopodia formation, cyclin B expression, and cell division by Etn. Anti-sense oligonucleotide against PKCβII or chlerythrine, a PKC specific inhibitor [Herbert et al., 1990] blocked Etn-induced filopodia formation 6 h after stimulation while control, mismatched oligonucleotide did not (Fig. 9A), indicating that PKCβII mediates Etn-induced cytoskeleton rearrangement. Furthermore, chlerythrine suppressed the expression of cyclin B and PKCβII, but did not influence the expression of PKCα and PKCζ (Fig. 9B). Chlerythrine also inhibited the inducible cell division (Fig. 9C). Unfortunately, we could not estimate the effect of antisense oligonucleotide on the cell division since the procedure of transfection itself inhibited cell

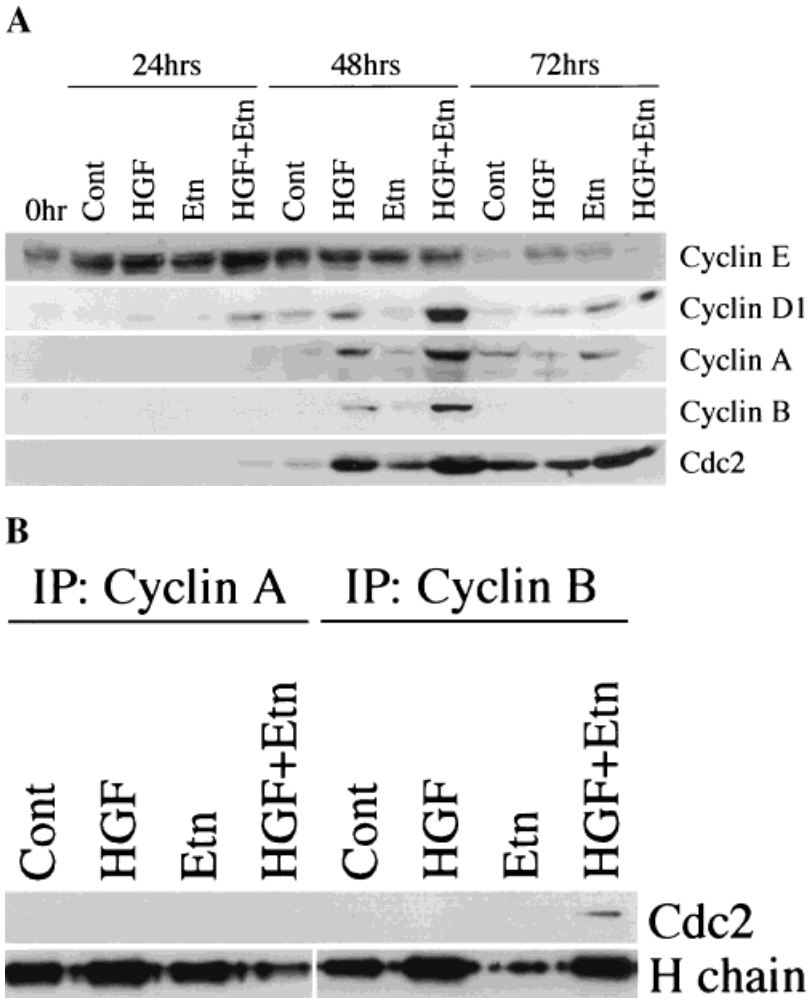


Fig. 5. Ethanolamine and HGF synergistically induce active cyclin B expression in primary hepatocytes. Primary hepatocytes were stimulated with Etn (100 μM) and HGF (10 ng/ml) for various time intervals then the cell lysates were subjected to Western blotting for cyclins and Cdc2 expression (**A**) and immunoprecipitation (**B**). B: Cell lysates were precipitated with anti-cyclin A or anti-cyclin B antibody and detected with anti-Cdc2 antibody as described in Materials and Methods.

division in positive control hepatocytes (data not shown).

Phosphatidylethanolamine (PE) Does not Mediate Etn-Inducible Effects on Hepatocytes

Etn is the first precursor molecule for the biosynthesis of PE and is converted to PE by cellular enzymes. Previous studies showed that PE increases cell proliferation [Kano-Sueoka et al., 1983; Kano-Sueoka and King, 1987] and is an essential molecule for cytokinesis during cell division [Emoto et al., 1996; Emoto and Umeda, 2000]. Thus, we examined whether the effects of Etn on hepatocytes are mediated through increased intracellular PE levels. Addition of Etn into the medium increased the amount of PE in primary hepatocytes as expected while that of phosphatidylcholine (PC) did not change (Fig. 10A). However, PE-liposome, which has been reported to induce cell division in other cell types [Emoto and Umeda, 2000] did not induce any significant Etn-like effects (filopodia formation or cell proliferation in primary hepatocytes) (Fig. 10B,D). Although we detected negligible expression of cyclin B by PE-liposome in the presence of HGF (Fig. 10C), the expression did not appear to affect cell proliferation (Fig. 10D). Taken together, these data indicate that the effects of Etn on hepatocytes are not based on increased PE in the cells but that the effects of Etn are mediated through a pathway distinct from that of PE biosynthesis.

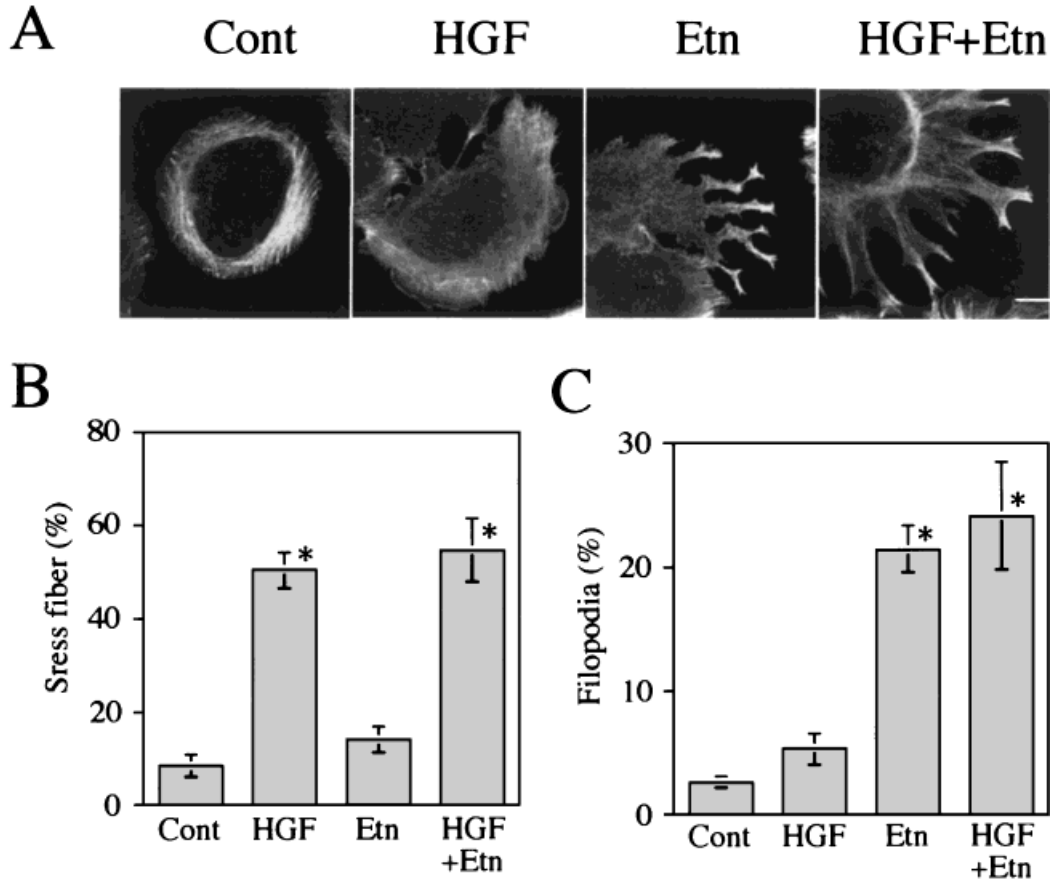


Fig. 6. Ethanolamine induces filopodia but not stress fiber formation in hepatocytes. Primary hepatocytes were stimulated by Etn (100 μM) and/or HGF (10 ng/ml) for 30 min or 6 h. **A**: Stimulated cells were stained with rhodamin-phalloidin after fixation and permealization, and then observed under a confocal microscope. Calibration bar, 20 μm. **B**: Cells with stress fiber formation were counted 30 min after stimulation. **C**: Cells with filopodia were counted 6 h after stimulation. $^*P < 0.02$.

DISCUSSION

The discrepancy between the ability of hepatocytes to proliferate in vivo and their inability to do so in vitro has been a challenging enigma for a number of hepatologists. Much attention has been paid to find G_1/S promoters while certain growth factors such as HGF, TGFα, and EGF have been reported to transit the cell cycle from G_1 to S phase in primary hepatocytes [Richman et al., 1976; McGowan et al., 1981; Mead and Fausto, 1989; Nakamura et al., 1989]. However, different from other cell types, primary hepatocytes do not proliferate in response to stimulation by only growth factors in vitro although they show increased DNA synthesis to such stimulation. Recent studies indicate that the transition processes from G_1 to S phase and from S to G_2/M phase are distinctly regulated by a variety of molecules such as cyclins [Nurse, 1990, 1994; Hunter and Pines, 1994; King et al., 1994]. For example, the transition from G_1 to S phase requires the complex formation of cyclin D_1/Cdk4 or 6, cyclin E/Cdk2, and cyclin A/Cdk2 [Hunter and Pines, 1994]. In contrast, for the transition to G_2/M phase, the complex formation of cyclin A/Cdc2 or cyclin B/Cdc2 is essential [Nurse, 1990, 1994; King et al., 1994]. In the cell cycle progression from G_1 to S phase of hepatocytes like other cell types, cyclin D_1 plays a critical role [Loyer et al., 1996; Kato et al., 1998; Albrecht and Hansen, 1999; Hansen and Albrecht, 1999]. However, the cell cycle mechanisms of primary hepatocytes to G_2/M phase progression are still unknown. Interestingly, p53-deficient hepatocytes different from other cell types do not proliferate in vitro although these cells are rarely arrested in G_1 [Tsukada et al., 1993]. Therefore, we hypothesized that there must be a restriction in G_2/M phase

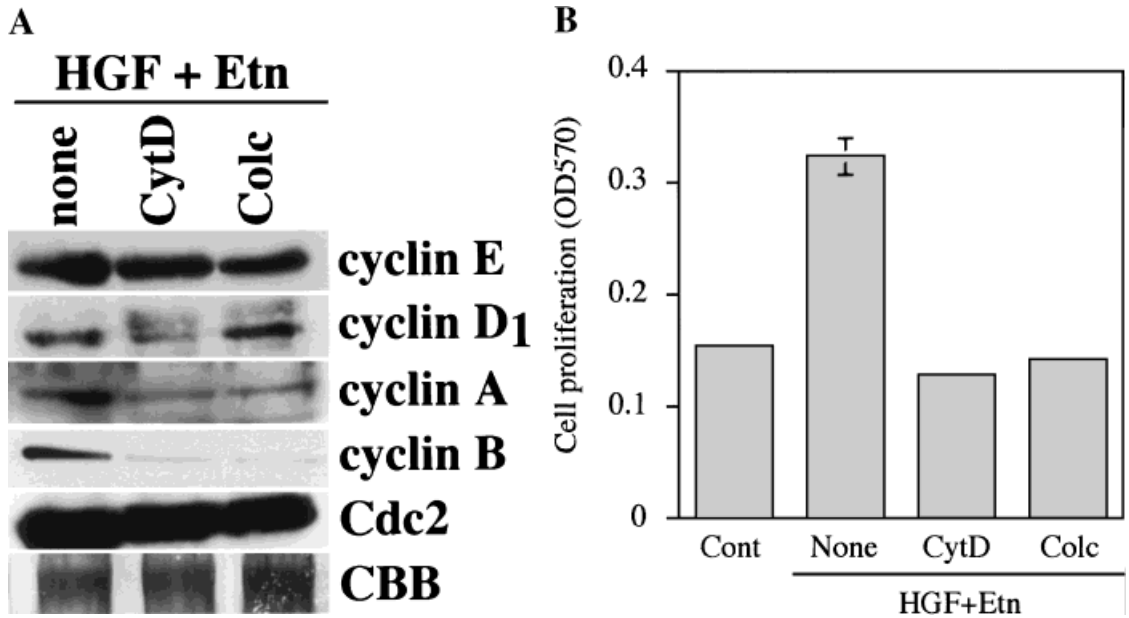


Fig. 7. Cytoskeleton rearrangement is required for both Etn-induced cyclin B expression and cell proliferation. Hepatocytes were cultured with Etn (100 μM) and HGF (10 ng/ml) in the presence and in the absence of cytochalasin D (CytD) (10 ng/ml) or colchicine (Colc) (40 μM). **A**: After 48 h of stimulation, the cell lysate was subjected to Western blotting for cyclin expression analysis as described. **B**: Cell proliferation assay was performed 96 h after stimulation.

transition including cytokinesis in primary cultured hepatocytes. The result that the addition of the conditioned medium to p53-deficient hepatocytes induced the proliferation strongly supported the hypothesis since these cells did not proliferate without CM even in the presence of growth factors (Fig. 2). In the present study, we identified Etn as a co-replicating factor for primary cultured hepatocytes.

Etn has been reported to enhance the G_1 progression in some cell types [Kano-Sueoka et al., 1983; Kano-Sueoka and King, 1987; Kiss and Crilly, 1996; Kiss et al., 1996, 1997a,b] including hepatocytes [Nelson et al., 1996; Sasaki et al., 1997]. In addition to this function, we demonstrated in the present study that Etn functions as a G_2/M promoter in primary hepatocytes. Etn with HGF induced cyclin A, B, and D_1 in primary hepatocytes (Fig. 5). In terms of cyclin D_1, Albrecht and Hansen [1999] elegantly demonstrated that the molecule is involved in the G_1/S transition of primary hepatocytes. We suggested in this study that the induced-cyclin B plays a critical role in the G_2/M transition of hepatocyte proliferation since the cyclin formed complex with Cdc2 in the stimulated hepatocytes. However, we can not rule out the possibility that cyclin A might be also involved in the regulation in some other way.

Interestingly, the medium (HGM) reported by Block et al. [1996] to enhance the proliferation of primary hepatocytes does not contain exogenous Etn. However, since their culture was relatively long-term, it is likely that dead cells released Etn from cytosol into the medium or that cells produced Etn by degrading endogenous PE with the activation of phospholipase D (PLD) [Kiss et al., 1997a]. It is also reported that the amount of Etn in the liver increases after partial hepatectomy in vivo [Houweling et al., 1992], and exogenously added Etn promoted liver regeneration [Sasaki et al., 1997]. These data suggest that Etn also functions as a co-mitogenic factor under physiological conditions. However, the mechanism that allows the entry of exogenously added Etn into hepatocytes remains unresolved. Taking the hydrophilicity of the molecule into consideration, it is likely that receptors or transporters for Etn exist on such cells. These acceptors should be identified in future studies. Etn is converted to PE in cells. PE has been reported to activate PKC [Bazzi et al., 1992] and be involved in cytokinesis [Emoto et al., 1996; Kiss et al., 1997a; Emoto and Umeda, 2000]. However, our

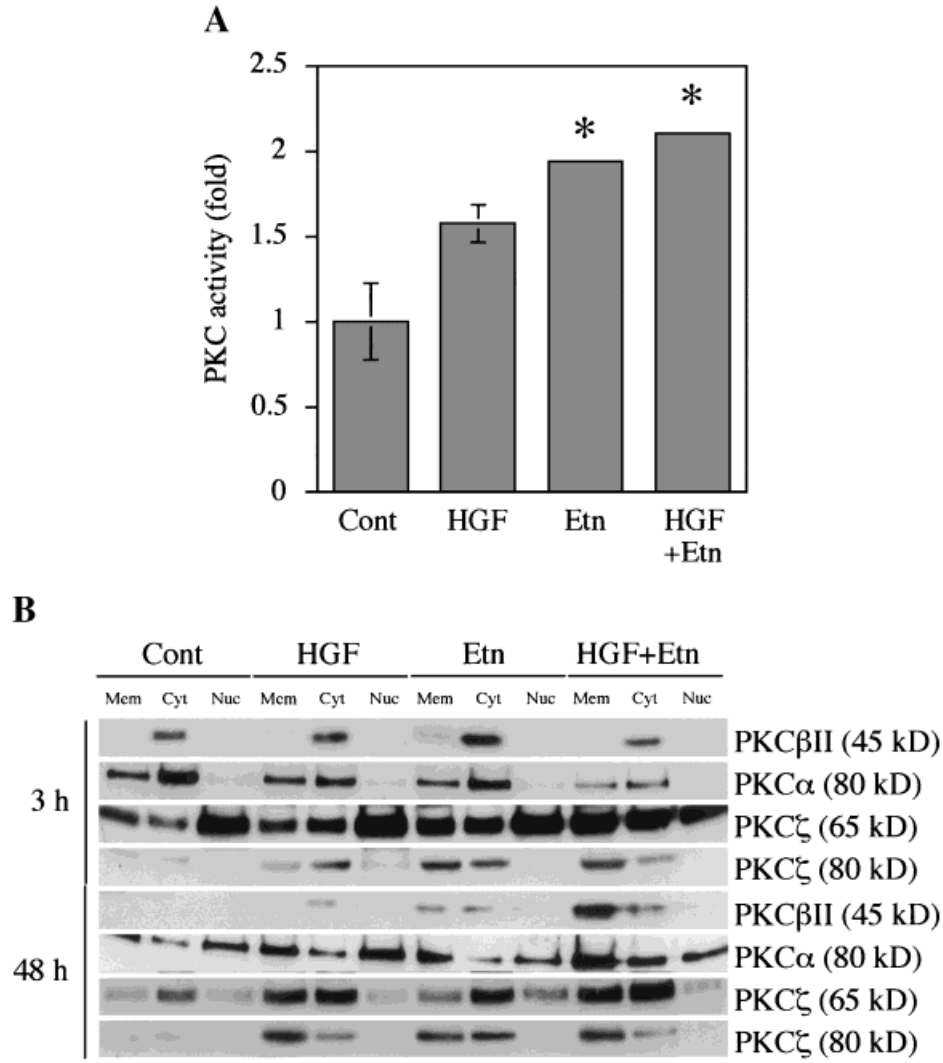
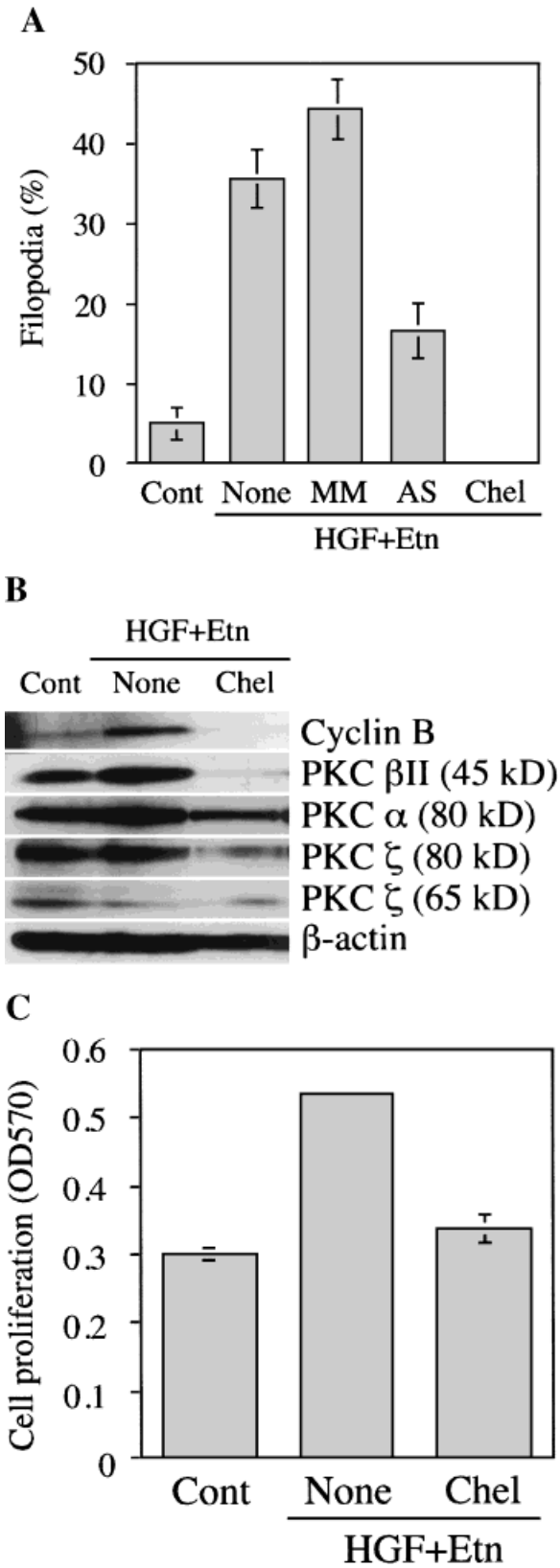


Fig. 8. Ethanolamine induces translocation of PKCβII to plasma membrane and enhances its expression. Primary hepatocytes were stimulated with Etn (100 μM) and HGF (10 ng/ml) for 30 min. **A**: 3 or 48 h (**B**). A: PKC activities in cell lysates were measured as described. B: Membrane (Mem), cytosol (Cyt), and nuclear (Nuc) fractions were isolated and subjected to Western blotting for various PKCs. Note that PKCβII translocated from the cytosol to membrane fraction and the expression increased at 48 h by Etn and HGF stimulation.

results strongly suggest that PE does not appear to mediate the effects of Etn on primary hepatocytes.

Cell shape and cytoskeleton organization regulate cell cycle [Huang and Ingber, 1999; Bar-Sagi and Hall, 2000]. Our data demonstrated that Etn regulates cell cycle via cytoskeleton reorganization represented by filopodia formation. The arrangement of cytoskeleton is controlled by Rho GTPase family, and Cdc42 is responsible for the filopodia formation [Bar-Sagi and Hall, 2000; Scita et al., 2000]. In addition, it has been reported that Cdc42 controls cell proliferation via JNK/ERK activation pathways [Coso et al., 1995; Minden et al., 1995; Bar-Sagi and Hall, 2000] and that PKC isoforms, PKCζ and PKCλ regulate cytoskeleton rearrangement by binding to Cdc42 [Überall et al., 1999; Coghlan et al., 2000]. Consequently, Cdc42 is thought to be involved in Etn-induced PKCβII-dependent cascades. However, since we were unable to detect a direct interaction between Cdc42 and PKCβII, it is likely that there are intermediate molecules that transduce the signal between them. For example, Tiam1, a guanine nucleotide exchange factor,



transduces the activation signal from PKC to Rac [Fleming et al., 1997]. The interaction between PKCβII and Cdc42 remains to be resolved.

PKCβII is one of the classical PKC family members and regulates the cell proliferation at G_2/M in some cell types [Hocevar and Fields, 1991; Murray et al., 1993; Goss et al., 1994; Thompson and Fields, 1996; Livneh and Fishman, 1997]. It has been reported that the PKCβII isoform (80 kDa) translocates into the nucleus and induces the disassembly of nuclear membrane at mitosis [Thompson and Fields, 1996; Livneh and Fishman, 1997]. However, our data in the present study demonstrated that Etn induced translocation of PKCβII from cytosol to plasma membrane in hepatocytes (Fig. 8). The PKCβII in hepatocytes is 45 kDa, which has so far been detected only in hepatocytes (our data and Ducher et al. [1995]) and is different from the previously reported one (80 kDa). PKCβII (45 kDa) was detected by an antibody against the C-terminal peptide and the expression was blocked by an anti-sense oligonucleotide against +31–48 bp. Thus, the PKCβII (45 kDa) detected in hepatocytes may be a novel, unknown splicing isoform of PKCβII, which might explain the contradictory behaviors. In this regard, PMA decreased the expression of PKCβII (80 kDa) [Murray et al., 1993] but not PKCβII (45 kDa) (data not shown). The translocation of PKCβII (45 kDa) to membrane is thought to be involved in the formation of filopodia. Our results also showed that Etn-induced PKCβII expression was suppressed by chlerythrine (Fig. 9). Since chlerythrine induces specific degradation of activated PKC [Thompson and Fields, 1996], the translocation and sensitivity to chlerythrine demonstrate the specific activation of PKCβII by Etn in hepatocytes. However, it is still unknown whether PKCβII is the direct target molecule of Etn.

Fig. 9. PKCβII mediates the effects of ethanolamine on filopodia formation, cyclin B expression, and cell proliferation. **A**: Isolated hepatocytes were transfected with antisense oligonucleotide (AS) against PKCβII or mismatched oligonucleotide (MM). These cells were stimulated with or without Etn (100 μM), HGF (10 ng/ml), or chlerythrine (Chel) (7 μM) 16 h after transfection and cultured for 6 h. The cells were stained with rhodamin-phalloidin after fixation, and filopodia formation was evaluated. **B**: Cell lysates were harvested from the transfected cells, and cyclin B and PKCs expression were analyzed by Western blotting. **C**: Hepatocytes were cultured with or without Etn (100 μM), HGF (10 ng/ml), or chlerythrine (7 μM) for 96 h and cell growth was evaluated by crystal violet assay.

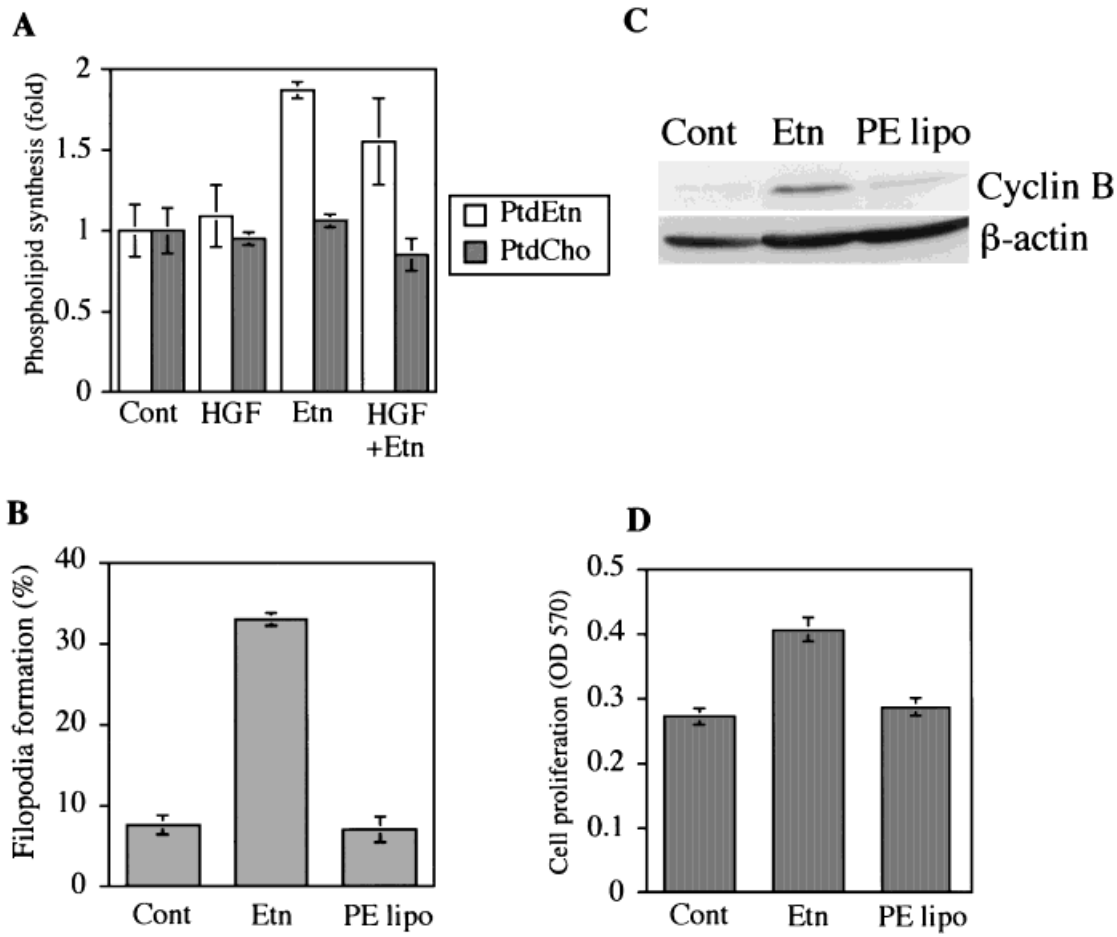


Fig. 10. Phosphatidylethanolamine is not the mediator of filopodia formation, cyclin B expression, or cell division by ethanolamine. **A**: Phospholipid contents in hepatocytes stimulated by Etn (100 mM) and/or HGF (10 ng/ml) were measured by TLC analysis as described. **B–D**: Hepatocytes were stimulated with Etn or PE-liposome (PE/PC = 1:1) in the presence of HGF (10 ng/ml). B: Filopodia formation was evaluated by staining cells with rhodamin-phalloidin and counted. C: Inducible cyclin B expression was examined in hepatocytes 48 h after the stimulation. D: Cell proliferation 96 h after the stimulation was estimated by crystal violet assay.

Further studies including finding the other specific activators of PKCβII are necessary to elicit the detail mechanism (s).

In conclusion, we shed some light on the role of Etn in hepatocyte proliferation by demonstrating that Etn activated PKCβII and translocated it to membrane, followed by filopodia formation and cyclin B expression. Further studies of Etn as a co-mitogenic factor in hepatocyte proliferation would enable the infinite passage of these cells in the future.

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