Ethanol potentiates the stimulatory effects of insulin and phosphocholine on mitogenesis by a zinc-dependent and rapamycin-sensitive mechanism in fibroblasts and JB6 cells

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In most cellular systems ethanol inhibits growth factor-induced cell growth. Here we examined the effects of ethanol on DNA synthesis and cell proliferation induced by insulin and phosphocholine (PCho) in NIH 3T3 fibroblasts, Swiss 3T3 fibroblasts and mouse epidermal JB6 cells. In serum-starved low (12–18) passage NIH 3T3 fibroblasts, 60 mM ethanol enhanced the mitogenic effect of insulin in the absence or presence of 25 μ M zinc about 2- or 12-fold, respectively. In contrast, in serum-starved high (30–47) passage NIH 3T3 cells 60 mM ethanol had large (20–40-fold) potentiating effects on insulin-induced DNA synthesis even in the absence of zinc. Furthermore, ethanol also enhanced the effects of PCho on DNA synthesis in both the absence and presence of insulin. The potentiating effects of ethanol on insulin- and PCho-induced DNA synthesis were associated with 1.2–1.3-fold stimulation of cell proliferation.

Rapamycin, an inhibitor of p70 S6 kinase action, strongly inhibited the potentiating effects of ethanol on insulin- and PCho-induced mitogenesis. Unexpectedly, ethanol inhibited synergistic activation of p42/p44 mitogen-activated protein kinases by insulin and PCho. In both Swiss 3T3 and JB6 cells, ethanol potentiated insulin-induced DNA synthesis only in the presence of zinc. In these cells, ethanol also increased the effects of PCho on both DNA synthesis and cell proliferation in the co-presence of either insulin or ATP. The results indicate that in various cell lines physiologically relevant concentrations of ethanol can increase the ability of insulin and PCho to induce DNA synthesis and, to smaller extents, cell proliferation. In low passage NIH 3T3 cells as well as in Swiss 3T3 and JB6 cells potentiation of insulin-induced DNA synthesis by ethanol requires the presence of zinc.

INTRODUCTION

A large number of studies indicate that ethanol inhibits liver regeneration as well as the growth of many different cell types examined under the conditions of cell culture [1–21]. Although there may not be a simple mechanism accounting for all the growth inhibitory effects of ethanol, inhibition of insulin-like growth factor I- and insulin receptor substrate-1-mediated signal transduction [18–21], and stimulation of translocation of cyclic AMP-dependent protein kinase catalytic subunit to the cell nucleus [22] may play important roles. However, all these effects of ethanol, like the effects of most growth regulatory agents, may depend on the cell type.

Indeed, in some cases ethanol actually has been found to exert positive effects on cell growth. These effects include stimulation of neurite growth in several brain regions as well as in cultured cerebral neurons and PC12 cells (referenced in [23]). In addition, ethanol was also shown to stimulate the proliferation of sub-ventricular cells in fetal cerebral cortex [24]. The molecular mechanism responsible for these growth stimulatory effects of ethanol is not clear. However, ethanol was shown to interact with the α -isoform of protein kinase C (PKC) [25], to increase the cellular level of PKC- ϵ and PKC- δ [26], to decrease phorbol ester-induced down-regulation of PKC- ϵ [27], and to increase the activity of p42/p44 mitogen-activated protein kinases (MAP kinases; also called extracellular signal-regulated kinases, or ERKs) [23].

Recently, we also reported [28] that in serum-starved NIH 3T3 fibroblasts, used at relatively low passages, ethanol was able to enhance modestly the stimulatory effects of insulin and insulin-

like growth factor, but not that of platelet-derived growth factor, fibroblast growth factor, or lyso-phosphatidic acid, on DNA synthesis. In subsequent experiments the potentiating effect of ethanol on insulin-induced DNA synthesis showed some variation. The pattern of variability appeared to relate, at least in part, to changes in the passage number. Therefore, we decided to re-examine the effects of ethanol on DNA synthesis in relatively low (12–18) and high (30–47) passages of NIH 3T3 fibroblasts as well as in Swiss 3T3 fibroblasts and mouse epidermal JB6 cells. An additional goal was to determine the possible effects of ethanol on cell proliferation.

Our recent work indicated that the effect of insulin on DNA synthesis may depend on the extracellular concentration of phosphocholine (PCho), ATP and zinc [29]. For this reason, we also examined the effects of ethanol on DNA synthesis in the presence of these agents. We have found that in each cell line used in this work, physiologically relevant concentrations of ethanol can significantly increase insulin- or insulin plus PCho-induced DNA synthesis, and that in some cases the potentiating effect of ethanol requires the presence of zinc. These co-mitogenic effects of ethanol, mediated by a rapamycin-sensitive MAP kinase-independent mechanism, were associated with relatively smaller increases in cell numbers.

EXPERIMENTAL

Materials

Seven different (1–7) preparations of ethanol were used as follows: (1) 95 % ethanol was from U.P.S., (2) absolute ethanol

Abbreviations used: PKC, protein kinase C; MAP kinase, p42/p44 mitogen-activated protein kinase; MTT, (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCho, phosphocholine.

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(200 proof) was from McCormick Distilling Co., Inc., (3) was redistilled '2' ethanol, (4) spectrophotometric grade ethanol was from Aldrich, (5) was redistilled '4' ethanol, (6) HPLC grade ethanol was from Aldrich, and (7) was redistilled '6' ethanol. The first ~ 35 % fraction of the distillate was always discarded. Unless specified, in most experiments the no. 5 ethanol preparation was used, and the results were confirmed with no. 3 and 1 ethanol preparations. Insulin was purchased from Boehringer-Mannheim; PCho, ATP, (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dowex-50W-H⁺ were bought from Sigma; [*methyl*-³H]thymidine (500 mCi/mmol) and [*methyl*-¹⁴C]choline chloride (50 mCi/mmol) were purchased from DuPont-NEN; and tissue culture reagents were purchased from Gibco-BRL.

Cell culture

NIH 3T3 clone-7 and Swiss 3T3 fibroblasts, both obtained from American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium as described earlier [29]. At the time of receiving NIH 3T3 and Swiss 3T3 fibroblasts they already were at passages 126 and 112, respectively, and they were frozen at passages 128 and 114, respectively. For these series of experiments NIH 3T3 cells were thawed and then continuously cultured for up to 47 passages (two series, started 3 months apart, were used), while Swiss 3T3 cells were used at 6–12 and 33–35 passages. For reasons of simplicity, in this work passages 128 and 114 were considered to be '0' passage. The mouse epidermal JB6 clone-41 cells, kindly provided by Dr. Nancy H. Colburn (National Institutes of Health, Frederick, MD, U.S.A.), were maintained in Eagle's minimum essential medium containing 10 % fetal calf serum as previously indicated [30]; these cells were used at 6–12 and 31–37 passages.

Labelling of cellular DNA with [³H]thymidine

The cells were grown in 12-well tissue culture dishes to about 40–50 % confluency in the presence of 10 % serum, washed, and then incubated in serum-free medium for 24 h. The cells (70–80 % confluent) were washed again and then treated (in serum-free medium; incubation volume, 0.75 ml) with ethanol, insulin and other agents as indicated for 16 h. Unless indicated otherwise, ethanol was added 20 or 10 min prior to insulin or PCho respectively. ATP was added 10 min prior to PCho, and 20 min prior to insulin. Finally, zinc chloride (25 μ M) was added 3 h prior to other additions, and it remained in the incubation medium during the 16 h treatment period. After treatments, incubations were continued in the presence of [*methyl*-³H]thymidine (1 μ Ci/well) for 60 min. The cells were washed twice with phosphate-buffered saline, then four times with 5 % trichloroacetic acid, and finally twice with absolute ethanol. The acid-insoluble material was redissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated ³H activity in a liquid scintillation counter. The cell number in ~ 80 % confluent cultures was ~ (1.5–1.6) $\times$ 10⁵/well.

Determination of ethanol effects on cell proliferation

NIH 3T3 cells (at passage 31–34) were seeded at 4000 cells/well in 96-microwell plates in serum-containing medium. After 24 h, the medium was replaced with serum-free medium and incubations were continued for 24 h. The cells were washed again, and then incubated for 2 days (in serum-free medium; incubation volume, 120 μ l) in the absence or presence of 60 mM ethanol and other additions as indicated in Table 3. The relative changes in the number of viable cells were determined by using the MTT

method [31] with the modifications described by Carmichael et al. [32], based on the measurement of formazan production. A Labsystem Multiskan MS microplate reader equipped with software and computer was used to read the absorbance using a test wavelength of 540 nm and a reference wavelength of 690 nm. Data are presented as the differences between absorbance values at 540 and 690 nm. To confirm the effects of ethanol on the number of viable cells, in some experiments cells were suspended by trypsin and quantified hemocytometrically.

Determination of ethanol effects on the phosphorylation of p42/p44 MAP kinases

NIH 3T3 cells (passage 31–34), grown in 6-well plates to about 80 % confluency, were treated first with 60 mM ethanol for 20 min followed by the addition of insulin for a 10 min incubation period. When applicable, 1 mM PCho was added 10 min prior to insulin. Samples for immunoblot analysis were prepared as described earlier [33]. Phosphospecific MAP kinase antibody (New England Biolabs), which recognizes the tyrosine-204 phosphorylation site, was used to detect the phosphorylated (activated) forms of p42 and p44 MAP kinases. The Western immunoblotting protocol was performed according to the instructions provided by the manufacturer.

Measurement of [¹⁴C]PCho formation and release

NIH 3T3 fibroblasts were grown in 12-well plates first in the presence of 10 % serum and then for 24 h in the absence of serum. Confluent fibroblasts were then incubated in the presence of 60 mM ethanol (1–7 preparations) and [¹⁴C]choline (0.5 μ Ci/well, 20 μ M) for up to 4 h. At the conclusion of incubation, the incubation medium (0.6 ml) was harvested (added to tubes containing 2 ml chloroform), and the cells were scraped into ice-cold methanol, followed by the rapid transfer of methanol extracts to tubes containing 2 ml of chloroform. [¹⁴C]PCho was separated from other metabolites on Dowex-50W-H⁺ columns as described previously [29].

Statistical methods

The unpaired *t* test was used to evaluate data from different experiments, while Student's *t* test was used to analyse data usually derived from six independent samples in the same experiment. *P* values of < 0.05–0.01 were considered significant.

RESULTS

Zinc-dependent synergic effects of ethanol and insulin on DNA synthesis in low passage NIH 3T3 cells

In our previous report [28], describing 2- to 3-fold potentiating effects of ethanol on insulin-induced DNA synthesis, NIH fibroblasts were used between 9 and 20 passages (passage number is defined in the Experimental section). In the present work, these cells (continuously passaged) were used either between 12 and 18 passages (considered low passages) or between 30 and 47 passages (considered high passages). One experiment was performed with passage 26 cells; in these cells the effects of ethanol were intermediate compared with its effects in the low and high passage categories.

In low passage cells, 40–60 mM concentrations of ethanol (no. 5 preparation) enhanced insulin-induced DNA synthesis only about 1.7–2-fold (Figure 1). In two other experiments performed with low passage cells, the small potentiating effect of 60 mM ethanol showed little variation (between 2.1- and 2.6-fold). In contrast, after pretreatment of cells with zinc, 40 and 60 mM

Figure 1 Zinc-dependent potentiating effect of ethanol on insulin-induced DNA synthesis in low passage NIH 3T3 cells

Serum-starved (24 h) passage-18 NIH 3T3 cells were untreated (open symbols) or treated (closed symbols) with 25 μ M zinc for 3 h, followed by treatments for 16 h with 0–60 mM concentrations of ethanol (no. 5 preparation) in the absence ($\circ$, $\bullet$) or presence (Δ , $\blacktriangle$) of 500 nM insulin. Each point represents the mean $\pm$ S.E.M. of three independent experiments each performed in triplicate with 1 day difference. * Significantly ($P < 0.01$) different from the corresponding value obtained in the absence of zinc (in the presence of insulin).

Table 1 Potentiating effects of 60 mM ethanol on PCho- and insulin-induced DNA synthesis in low passage NIH 3T3 cells

Serum-starved passage-16 NIH 3T3 fibroblasts were treated with 60 mM ethanol (no. 5 preparation) for 16 h in the absence or presence of 25 μ M zinc, 1 mM PCho, 0.1 mM ATP, and/or 500 nM insulin as indicated; zinc was added to the cells 3 h prior to ethanol. The data are the means $\pm$ S.D. ($n = 6$), d.p.m./well per 60 min. Similar results were obtained in two other experiments performed in triplicate with passage-17 and -19 cells.

Addition	Incorporation of [3 H]thymidine into DNA			
	No zinc		Plus zinc	
	No ethanol	Plus ethanol*	No ethanol	Plus ethanol*
None	930 $\pm$ 160	8730 $\pm$ 610	2540 $\pm$ 350	15210 $\pm$ 1760
PCho	7370 $\pm$ 590	28830 $\pm$ 2610	18810 $\pm$ 3010	62550 $\pm$ 3580
PCho + ATP	34550 $\pm$ 4490	62770 $\pm$ 5040	51320 $\pm$ 4360	83470 $\pm$ 6160
Insulin	12010 $\pm$ 1620	21320 $\pm$ 2970	15490 $\pm$ 2050	120590 $\pm$ 12110
PCho + insulin	235310 $\pm$ 24680	331940 $\pm$ 20280	249410 $\pm$ 19180	324380 $\pm$ 24290

* In each case the effect of ethanol was significantly ($P < 0.01$ level) different from that observed in the corresponding treated (ethanol-free) samples.

concentrations of ethanol increased the stimulatory effect of insulin 3.7- and 20-fold, respectively (Figure 1). The potentiating effect of zinc was reproducible when it was added prior to ethanol. The length of the preincubation period minimally required for the full effect of zinc remains to be determined. It still worth noting that in the presence of zinc, ethanol had

Figure 2 Similar potentiating effects of seven different ethanol preparations on insulin-induced DNA synthesis in high passage NIH 3T3 cells

Serum-starved (24 h) passage-30 NIH 3T3 cells were treated for 16 h with 40–80 mM concentrations of no. 5 ethanol in the absence of insulin ($\circ$); in all the other cases, when cells were treated with ethanol 1 ($\bullet$), ethanol 2 (Δ), ethanol 3 ($\blacktriangle$), ethanol 4 ($\square$), ethanol 5 ($\blacksquare$), ethanol 6 ($\diamond$), and ethanol 7 ($\blacklozenge$) preparations, 500 nM insulin was included. The results are the mean of three independent incubations. The difference between the extreme values was always less than 14%. Similar results were obtained in another experiment performed in triplicate.

detectable (4–7-fold) stimulatory effects on DNA synthesis even in the absence of insulin (Figure 1).

Combined stimulatory effects of ethanol, insulin, PCho, ATP and zinc on DNA synthesis in low passage NIH 3T3 cells

In an experiment performed with passage-16 cells 3 months after the experiment described in Figure 1 was performed, ethanol (no. 5 preparation) again had only a relatively small (~ 1.8 -fold) potentiating effect on insulin-induced DNA synthesis unless zinc was also present (Table 1). However, in these cells ethanol considerably (3.3–3.8-fold) increased PCho-induced DNA synthesis in both the absence and presence of zinc (Table 1). In these low passage cells PCho and insulin had very large synergistic effects on DNA synthesis which was only marginally (~ 1.2 – 1.3 -fold) increased by ethanol. In accordance with previous data [29], PCho and ATP also synergistically enhanced DNA synthesis. The combined effects of PCho and ATP were only slightly increased by ethanol in both the absence and presence of zinc (Table 1).

Synergistic effects of ethanol and insulin on DNA synthesis in high passage cells in the absence of added zinc

In the following experiments performed with high (30–47) passage cells, seven different preparations of ethanol were used (as

Figure 3 Zinc further increases the potentiating effect of ethanol on insulin-induced DNA synthesis in high passage NIH 3T3 cells

Serum-starved passage-36 NIH 3T3 cells were untreated (**A**) or were treated with 25 μ M zinc for 3 h (**B**), followed by treatments for 16 h (in the continuous presence of zinc if applicable) with 0–80 mM concentrations of no. 5 preparation of ethanol in the absence (●) or presence (▲) of 500 nM insulin. Each point represents the mean $\pm$ S.D. ($n = 6$). Similar results were obtained in two other experiments, each performed in triplicate, with no. 1 and 3 ethanol preparations, respectively. * Significantly different from untreated control, and ** significantly different from values obtained in the presence of ethanol alone ($P < 0.01$).

described in the Experimental section) to verify that its potentiating effects were not caused by some contaminant. At 60 and 80 mM concentrations, ethanol alone (as an example, only the effect of preparation 5 is shown) stimulated DNA synthesis (Figure 2); there were no major differences in the effects of different ethanol preparations. In contrast with the low passage cells, in the high passage cells 60 and 80 mM concentrations of ethanol greatly enhanced the effect of insulin on DNA synthesis even in the absence of added zinc. The maximal effects of 80 mM ethanol in the presence of insulin were about 60–75% of that which can be induced by 10% serum. While each ethanol preparation had large potentiating effects, the no. 2 preparation was somewhat less effective than the other preparations. However, distillation restored the activity of this ethanol preparation (Figure 2), suggesting that the commercially available form of no. 2 ethanol may contain an inhibitor of ethanol effect. Overall, the results indicated that in NIH 3T3 cells elevation of ethanol concentration from 40 to 60 mM can cause dramatic potentiating effects on insulin-induced signal transduction, depending on the passage number and on the presence of zinc. In the following experiments, aimed at better understanding of these ethanol effects, the no. 5 ethanol preparation was used unless indicated otherwise.

Effects of zinc on the stimulatory actions of ethanol on DNA synthesis in high passage cells

Although in high passage cells 60 and 80 mM concentrations of ethanol significantly enhanced the effect of insulin on DNA synthesis even in the absence of zinc (Figure 2 and Figure 3A), zinc was able to further increase the effects of ethanol (Figure 3B). For example, in the presence of zinc the potentiating effect of 40 mM ethanol on insulin-induced DNA synthesis was in-

creased about 6-fold, while the effects of 40–80 mM concentrations of ethanol in the absence of insulin were increased 4.5–10-fold (Figure 3B compared with Figure 3A).

Modification of the stimulatory ethanol effects on DNA synthesis by the length of preincubation period as well as by rapamycin and wortmannin

In passage 39 NIH 3T3 cells ethanol was most effective in potentiating insulin-induced DNA synthesis when presented to the cells simultaneously with insulin (~ 15 -fold increase), but it still had considerable (~ 10 -fold) potentiating effect when added 60 min prior to insulin (Figure 4). In the same experiment, ethanol was found to exert comparable potentiating effects when it was added simultaneously or 10 min after insulin (results not shown). Rapamycin, a specific inhibitor of p70 S6 kinase action [34,35], was previously shown to inhibit the mitogenic effects of insulin in the presence of PCho [29]. As shown in Figure 4, rapamycin also strongly inhibited the combined stimulatory effects of ethanol and insulin on DNA synthesis. Since insulin stimulation of p70 S6 kinase is mediated by phosphatidylinositol 3'-kinase [34,36], we also examined the effect of wortmannin, an inhibitor of the latter enzyme [37,38], on the combined effects of ethanol and insulin on DNA synthesis. Wortmannin (100 nM), added 30 min prior to ethanol (60 mM) and 50 min prior to insulin, inhibited insulin-induced DNA synthesis in the absence and presence of ethanol by ~ 60 and 45%, respectively.

Effects of ethanol on DNA synthesis in high passage NIH 3T3 cells stimulated by PCho, PCho plus insulin, or PCho plus ATP

As previously reported [29], in NIH 3T3 cells PCho (0.25–1 mM) was a relatively weak mitogen in the absence of ATP or insulin.

Figure 4 The potentiating effect of ethanol on insulin-induced DNA synthesis does not require preincubation and is inhibited by rapamycin in NIH 3T3 cells

Serum-starved passage 39 NIH 3T3 cells were first incubated for 10 min in the absence (●) or presence (▲) of 10 nM rapamycin, followed by treatments with 60 mM ethanol (no. 5 preparation) for 0–60 min, as indicated, prior to the addition of 500 nM insulin; (○) indicates the effect of insulin alone. Each point represents the mean $\pm$ S.D. ($n = 3$). Similar results were obtained in another experiment performed in triplicate with passage-40 cells.

However, in high passage (33) NIH 3T3 cells its effects were greatly enhanced by both ethanol and zinc (Figure 5). Ethanol and zinc had some synergistic effects at 0.5 mM, but not at other concentrations of PCho (Figure 5). For this reason, in the next experiment PCho was used at 0.5 mM to determine whether its combined effects with that of ATP and insulin can be modified by ethanol.

In agreement with our previous findings [29], ATP and PCho synergistically stimulated DNA synthesis in the absence of ethanol, but the potentiating effect of ethanol on PCho-induced DNA synthesis was not significantly modulated by ATP (Table 2). In contrast, the potentiating effect of ethanol on insulin-induced DNA synthesis was further enhanced by 0.5 mM PCho (Table 2).

Combined effects of ethanol, insulin, and PCho on cell proliferation and MAP kinase activity in high passage NIH 3T3 cells

In high passage (31) NIH 3T3 cells (Table 3) insulin and 1 mM PCho had less robust synergistic effects on DNA synthesis than in low passage cells (Table 1). Conversely, in high passage cells ethanol considerably (2.8-fold) enhanced the combined effects of PCho and insulin as well as the individual effects of PCho and insulin on DNA synthesis (Table 3). After treatment of passage 31 cells with insulin plus PCho for 2 days, there was an about 1.3–1.4-fold increase in the proportion of viable cells determined either by the MTT assay (Table 3) or by direct counting (results not shown). With either method, 60 mM ethanol enhanced both the combined effects of insulin and PCho (~ 1.25 -fold increase) and their smaller individual effects (1.17–1.3-fold increases) on

Figure 5 Synergistic stimulation of DNA synthesis by ethanol and PCho in NIH 3T3 cells

Serum-starved passage 38 NIH 3T3 cells were first incubated for 3 h in the absence (○, ●) or presence (△, ▲) of 25 μ M zinc, followed by treatments for 16 h with 0–1 mM concentrations of PCho in the absence (open symbols) or presence (closed symbols) of 60 mM no. 5 ethanol preparation. Each point represents the mean $\pm$ S.D. ($n = 6$). * Significantly different from the corresponding value obtained in the absence of ethanol and zinc, or ** significantly different from the value obtained in the absence of ethanol and in the presence of zinc.

Table 2 Potentiating effects of 60 mM ethanol on PCho- and insulin-induced DNA synthesis in high passage NIH 3T3 cells

Serum-starved passage-33 NIH 3T3 fibroblasts were treated with 60 mM ethanol (no. 5 preparation) for 16 h in the absence or presence of 25 μ M zinc, 0.5 mM PCho, 0.1 mM ATP, and/or 500 nM insulin as indicated. Zinc was added to the cells 3 h prior to other additions. The data are the means $\pm$ S.D. ($n = 6$), d.p.m./well per 60 min. Similar results were obtained in two other experiments each performed in triplicate.

Addition	Incorporation of [3 H]thymidine into DNA			
	No zinc		Plus zinc	
	No ethanol	Plus ethanol*	No ethanol	Plus ethanol*
None	450 $\pm$ 40	7610 $\pm$ 620	690 $\pm$ 75	39970 $\pm$ 1230
PCho	1950 $\pm$ 390	15250 $\pm$ 1150	5960 $\pm$ 490	56820 $\pm$ 3030
ATP + PCho	6360 $\pm$ 370	16480 $\pm$ 1970	10330 $\pm$ 1350	62920 $\pm$ 1950
Insulin	3620 $\pm$ 310	81650 $\pm$ 5910	4780 $\pm$ 570	120470 $\pm$ 7020
Insulin + PCho	5510 $\pm$ 480	116500 $\pm$ 7280	26610 $\pm$ 1090	149030 $\pm$ 10890

* In each case the effect of ethanol was significantly ($P < 0.01$ level) different from that observed in the corresponding treated (ethanol-free) samples.

cell proliferation during a 2-day incubation period (Table 3). While the effects of ethanol on cell numbers were statistically significant, these effects were clearly much smaller than the potentiating effects of ethanol on DNA synthesis. This suggested that ethanol may not activate an important component of the cell signalling mechanism required for cell cycle progression.

Activated p42/p44 MAP kinases are generally regarded as

Table 3 Combined stimulatory effects of ethanol, insulin, ATP and PCho on DNA synthesis and cell proliferation in high passage NIH 3T3 cells

For the study of DNA synthesis, serum starved passage 31 NIH 3T3 cells, grown in 12-well plates, were treated with 60 mM ethanol (no. 5 preparation), 1 mM PCho and/or 500 nM insulin, as indicated, for 16 h. For the study of cell proliferation by the MTT assay, serum-starved passage 31 NIH 3T3 cells, grown in 96-well plates, were treated with ethanol, PCho and insulin as indicated above for 48 h. The data are the means $\pm$ S.D. of eight incubations (MTT assay) or six incubations (DNA synthesis). Similar results were obtained in two other experiments each performed with passage-44 and -47 cells.

Addition	Incorporation of [3 H]thymidine into DNA		Viable cells; A_{540}	
	No ethanol	Plus ethanol	No ethanol	Plus ethanol
None	550 $\pm$ 110	910 $\pm$ 220	0.537 $\pm$ 0.040	0.548 $\pm$ 0.036
PCho	3950 $\pm$ 560	10250 $\pm$ 2150*	0.551 $\pm$ 0.030	0.659 $\pm$ 0.033*
Insulin	8820 $\pm$ 1410	78830 $\pm$ 4960*	0.610 $\pm$ 0.018	0.793 $\pm$ 0.050*
Insulin + PCho	52220 $\pm$ 5570	146280 $\pm$ 17110*	0.714 $\pm$ 0.029	0.911 $\pm$ 0.046*

* Significantly ($P < 0.05$ – 0.01) different from the value observed in the corresponding (ethanol-free) treated sample.

Figure 6 Effects of ethanol on the phosphorylation of MAP kinases in NIH 3T3 cells

Serum-starved passage 31 NIH 3T3 cells were first incubated for 10 min with no. 5 preparation of ethanol (60 mM) (lanes 5, 6), followed by incubations in the presence of 1 mM PCho for 10 min (lanes 4, 6), and then in the presence of 500 nM insulin for an additional 10 min (lanes 3–6). Lane 2 represents a non-treated incubated sample, while lane 1 represents the standard phosphorylated MAP kinases (the upper and lower band, best seen in lane 4, being represented by the p42 and p44 MAP kinases, respectively) analysed by immunoblotting. The arrow indicates the position of MAP kinases.

important regulators of both DNA synthesis and cell proliferation. MAP kinases are phosphorylated and thereby activated by MAP kinase kinases, which phosphorylate both tyrosine and threonine residues in p42/p44 MAP kinases [39]. To assess possible modulation of the activity state of p42/p44 MAP kinases, we next examined the effects of ethanol alone and in combination with insulin and PCho on tyrosine phosphorylation of these enzymes. As shown in Figure 6, in passage 33 serum-starved NIH 3T3 cells insulin and PCho in combination enhanced phosphorylation of MAP kinases. When added to the cells alone, insulin had only a very small effect (Figure 6), and PCho was without effect ([29]; not shown here). Surprisingly, 60 mM ethanol not only failed to promote the effect of insulin alone, but it actually inhibited the combined stimulatory effects of PCho and insulin on MAP kinase phosphorylation (Figure 6).

Effects of ethanol on DNA synthesis in Swiss 3T3 and JB6 cells

Since ethanol causes changes in the growth of many different cell types [1–21], it was of interest to determine whether the potentiating effect of ethanol on insulin- and PCho-induced DNA synthesis can also be observed in other cell lines. In low passage (6–12) Swiss 3T3 and JB6 cells, the two additional cell lines

Table 4 Stimulatory effects of ethanol on DNA synthesis in Swiss 3T3 and JB6 cells

Serum-starved passage-6 Swiss 3T3 cells and passage-7 JB6 cells were treated with 60 mM ethanol (no. 5 preparation) for 16 h in the absence or presence of 25 μ M zinc, 1 mM PCho, 500 nM insulin or 0.1 mM ATP. The data are the means $\pm$ S.D. ($n = 6$), d.p.m./well per 60 min. Similar results were obtained in another experiment ($n = 6$) performed with passage-9 cells.

Addition	Incorporation of [3 H]thymidine into DNA			
	Swiss 3T3		JB6	
	No ethanol	Plus ethanol	No ethanol	Plus ethanol
None	4020 $\pm$ 260	3960 $\pm$ 240	2420 $\pm$ 320	1860 $\pm$ 220
Zinc	3330 $\pm$ 290	4160 $\pm$ 350	2090 $\pm$ 220	2280 $\pm$ 130
Insulin	12960 $\pm$ 1080	9220 $\pm$ 810	151120 $\pm$ 11260	115920 $\pm$ 9700
Zinc + insulin	10450 $\pm$ 1140	24690 $\pm$ 2340*	156040 $\pm$ 14310	249030 $\pm$ 18610*
PCho	5960 $\pm$ 310	4520 $\pm$ 480	3520 $\pm$ 360	1900 $\pm$ 170
PCho + insulin	18540 $\pm$ 2020	68960 $\pm$ 8620*	241620 $\pm$ 16810	367500 $\pm$ 25580*
PCho + ATP	14770 $\pm$ 2490	46150 $\pm$ 3130*	6440 $\pm$ 410	58530 $\pm$ 5910*

* Significantly ($P < 0.01$) different from the value observed in the corresponding (ethanol-free) sample.

chosen for these studies, the stimulatory effects of insulin were inhibited, rather than increased, by 60 mM ethanol when zinc was absent from the incubation medium (Table 4). Importantly, in both cell lines ethanol clearly potentiated the effect of insulin in the presence of 25 μ M zinc (Table 4). Unlike in NIH 3T3 cells, in Swiss 3T3 and JB6 cells ethanol failed to enhance DNA synthesis in the presence of PCho alone (Table 4). In contrast, in both cell lines ethanol further enhanced the synergistic effects of PCho plus insulin and PCho plus ATP (Table 4). We also examined the combined effects of ethanol and insulin in Swiss 3T3 and JB6 cells at higher (31–37) passage numbers; in both cell lines, ethanol decreased or increased the effects of insulin on DNA synthesis in the absence or presence of zinc, respectively.

In Swiss 3T3 cells, the potentiating effects of various concentrations of ethanol on insulin plus PCho-induced DNA synthesis were slightly decreased by zinc (Figure 7A). In contrast, in JB6 cells zinc enhanced the combined effects of insulin and PCho by about 20 %, but the potentiating effects of ethanol were not significantly altered by zinc (Figure 7B). Similar to NIH 3T3 cells, ethanol increased, by about 15–23 %, the 1.3–1.4-fold stimulatory effects of PCho plus insulin on the proliferation of Swiss 3T3 and JB6 cells (results not shown).

Effects of ethanol on the formation and release of PCho

It was possible that, similar to ATP [29], ethanol stimulates the release of PCho from cells into the medium. Considering that PCho and insulin act synergistically on DNA synthesis via extracellular sites [29], released PCho could mediate the potentiating effect of ethanol on insulin-induced mitogenesis. We examined this possibility and found that ethanol modified neither the formation of [14 C]PCho from [14 C]choline nor the release of [14 C]PCho from cells into the medium (results not shown).

DISCUSSION

In view of the many earlier reports [1–21] showing that ethanol inhibits cell growth in most cell types examined, our previous finding [28] that in relatively low passage NIH 3T3 cells ethanol enhanced the effect of insulin on DNA synthesis was unusual. This potentiating effect of ethanol on DNA synthesis was

Figure 7 Effects of ethanol on DNA synthesis in Swiss 3T3 and JB6 cells

Serum-starved passage-6 Swiss 3T3 cells (A) and passage-7 JB6 cells (B) were treated with increasing concentrations (0–80 mM) of no. 5 preparation of ethanol in the absence (●) or presence of 25 μ M zinc (▲), 1 mM PCho plus 500 nM insulin (■), or zinc plus PCho plus insulin (◆). Each point represents the mean $\pm$ S.D. ($n = 6$). Similar results were obtained in another experiment performed with passage-12 Swiss 3T3 and JB6 cells under identical conditions.

observed only in the presence of insulin or insulin-like growth factor I, while the mitogenic effects of platelet-derived growth factor, fibroblast growth factor, or lysophosphatidic acid were not increased by ethanol [28]. Because insulin is involved in the regulation of growth of many different cell types, including transformed cells, we decided to examine this unique co-mitogenic effect of ethanol in more detail. In particular, we wished to determine the (i) optimal conditions for the co-mitogenic ethanol effects, (ii) the relationship between the effects of ethanol on DNA synthesis and cell proliferation, and (iii) the possible stimulatory effects of ethanol on mitogenesis in some other cell lines. In this effort, we compared the effects of ethanol on mitogenesis in relatively low and high passages of NIH 3T3 cells as well as in Swiss 3T3 and JB6 cells, and also analysed the dependence of ethanol effects on zinc, PCho and ATP. These agents were tested because in a previous work they were found to enhance the mitogenic activity of insulin [29].

The approach taken here resulted in several interesting observations. The first important finding was that in low passage NIH 3T3 cells the small potentiating effect of ethanol (60 mM) on insulin-induced DNA synthesis was sharply increased when the cells were pretreated with a physiological (25 μ M) concentration of zinc. Zinc had detectable, but significantly less, potentiating effect at 40 mM ethanol. To our best knowledge, this is the first description of an interaction between ethanol and zinc on DNA synthesis. Since zinc is one of the most important micronutrients [40,41], which is present in the circulation in the 10–35 μ M range [42–44], it is possible that the effects of ethanol on DNA synthesis *in vivo* may also depend on the zinc status.

In contrast with low passage cells, in high passage NIH 3T3 cells 40–60 mM concentrations of ethanol, which are physiologically relevant [45], significantly enhanced the stimulatory effect of insulin on DNA synthesis even in the absence of zinc. The reason why low passage cells required, and high passage cells apparently did not require, zinc for a significant ethanol action is presently unclear. Currently, we are examining the possibility that the actions of ethanol are zinc-dependent in both cases, but

high passage cells can retain more zinc for a longer time period than low passage cells. While the mechanism of zinc action remains to be determined, it is interesting to note that in cortical neurons inhibition of glutamate receptor-stimulated nitric oxide synthase by ethanol also requires zinc [46]. Thus, it appears that zinc has an important role in modulating various biological effects of ethanol.

Seven different preparations of ethanol, most of them highly purified, induced similar effects on DNA synthesis. This clearly indicates that ethanol itself, and not a contaminant, was the active agent. However, we cannot exclude the possibility that the co-mitogenic effects of ethanol were actually mediated by a rapidly formed metabolite. Clarification of this issue will require further experiments.

With each ethanol preparation, there was a sharp increase in the co-mitogenic effect of ethanol in the 40–60 mM concentration range. The mechanism accounting for the abrupt increase in ethanol effect in the relatively narrow concentration range is presently unknown. If the primary target were an enzyme or a receptor protein, one might expect a 'smoother' concentration-dependence of ethanol effect. Clearly, more work is needed to clarify the primary target of ethanol action and to determine the mode of interaction between ethanol and its primary target, presumably located in the plasma membrane.

In fibroblasts insulin alone is a poor mitogen. This is because insulin acts as a progression factor; thus, its full effect on mitogenesis requires the presence of inducers of the mitogenic event. In NIH 3T3 cells ethanol, like PCho, appears to act as an initiator of DNA synthesis, because in the presence of insulin it can increase DNA synthesis. However, if both PCho and ethanol act by initiating DNA synthesis which is then promoted by insulin, it is difficult to understand how ethanol and PCho are able to enhance each other's effects in the absence of a progression factor. One possibility is that ethanol increases the release of a progression factor, which then enhances the effect of PCho. It should be noted that in NIH 3T3 cells there is a relatively high basal level of ATP release [47], and this is greatly increased by even small stress [48]. Considering that ATP acts as a progression factor [29], and that it enhances the effects of PCho on DNA synthesis, ethanol may potentiate the effect of PCho by either stimulating the release of ATP from cells or by sensitizing the cells to the action of ATP. We are presently investigating both possibilities.

The findings that the positive effects of ethanol on mitogenesis are not confined to NIH 3T3 cells are also important. In Swiss 3T3 and JB6 cells, as in low passage NIH 3T3 cells, potentiation of insulin effect by ethanol required the addition of zinc to the incubation medium. Furthermore, in Swiss 3T3 and JB6 cells potentiation of PCho effect by ethanol required the simultaneous presence of either insulin or ATP, both apparently acting as progression factors. These data fit the emerging mechanism that in the presence of zinc ethanol can act as an initiator or co-initiator of mitogenesis, which can significantly increase DNA synthesis only if a progression factor (insulin, ATP, or yet another progression factor) is also present. In this context, it should be mentioned that in Swiss 3T3 cells ethanol was previously found by other investigators to inhibit the effect of insulin on DNA synthesis [18]. In agreement with this observation [18], we also found that in both Swiss 3T3 and JB6 cells ethanol inhibits the effect of insulin on DNA synthesis unless zinc is added to the incubation medium. As discussed above, the mechanism by which zinc can switch the inhibitory effect of ethanol to a stimulatory one remains to be determined.

It is presently unclear how ethanol can both enhance the effects of insulin and PCho on DNA synthesis and inhibit

activation of p42/p44 MAP kinases by these agents. These MAP kinases are reportedly required for G₀ to S phase transition [49], although bombesin and insulin are known to stimulate synergistically entry of Swiss 3T3 fibroblasts into S phase by a MAP kinase-independent mechanism [50]. Based on the inhibitory effects of rapamycin and wortmannin, it appears that the signal transduction pathway mediating the effects of ethanol and insulin on DNA synthesis in NIH 3T3 fibroblasts, and the combined effects of ethanol with PCho plus insulin or PCho plus ATP in Swiss 3T3 and JB6 cells involves the p70 S6 kinase and phosphatidylinositol 3'-kinase activities. However, additional growth regulatory mechanisms may also be required for increased DNA synthesis in cells stimulated by ethanol, insulin and PCho. In this context it should be mentioned that insulin has recently been shown to stimulate phosphorylation and activation of various STAT (signal transducers and activators of transcription) proteins [51–53]; thus, these proteins may also be targets of the combined actions of ethanol and insulin. Clearly, more experiments are required to determine the exact nature of signal transduction pathways involved in the mediation of insulin- and PCho-dependent effects of ethanol on cell growth.

An important aspect of ethanol effect on mitogenesis is that, despite its large stimulatory effects on DNA synthesis, it had only relatively small stimulatory effects on cell proliferation. This suggests that one or more of the important growth regulatory mechanisms, required for the transition of cells from the S phase to the next phases, is (are) adversely affected by ethanol. In view of the important role of p42/p44 MAP kinases in cell proliferation, the observed inhibitory effects of ethanol on insulin-plus-PCho-induced MAP kinase phosphorylation, and presumably on MAP kinase activity, may explain the discrepancy between the effects of ethanol on DNA synthesis and cell proliferation. However, a final conclusion must await a more complete understanding of ethanol effects on the various signal transduction mechanisms involved in the regulation of cell growth.

In summary, we have shown that in three cell lines ethanol is able to enhance DNA synthesis accompanied by modest increases in cell proliferation. Depending on the cell type and passage number, the stimulatory effects of ethanol on DNA synthesis were found to require the presence of insulin and/or PCho, ATP or zinc. Unexpectedly, the effects of ethanol on mitogenesis occurred in the absence of activation of p42/p44 MAP kinases. Further experiments are needed to determine the role of various signal transduction pathways in the mediation of co-mitogenic effects of ethanol and to evaluate the significance of the observed ethanol effects on mitogenesis *in vivo*.

This work was supported by NIH Grant AA09292.

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