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ETHANOL ENHANCES THE STIMULATORY EFFECTS OF INSULIN AND INSULIN-LIKE GROWTH FACTOR-1 ON DNA SYNTHESIS IN NIH 3T3 FIBROBLASTS

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SUMMARY: In practically all *in vitro* experimental systems examined so far, including embryonal fibroblasts, ethanol was shown to inhibit cell growth. Here we report that in NIH 3T3 fibroblasts, (patho)physiologically relevant concentrations (50-100 mM) of ethanol significantly (2- to 2.8-fold) enhanced the stimulatory effects of both insulin and insulin-like growth factor-1 on DNA synthesis. Ethanol had no major effects on the mitogenic effects of platelet-derived growth factor, fibroblast growth factor and lyso-phosphatidic acid. These data suggest that ethanol is not a universal inhibitor of cell growth.

In most cell types and tissues examined so far, ethanol appears to inhibit the growth-promoting effects of various growth factors, including those of epidermal growth factor in hepatocytes [1,2], IGF-1 in Balb/c 3T3 fibroblasts [3], insulin in rat liver [4], and serum in glioma-derived C_6 cells [5]. As a result, ethanol usually reduces the number of both neural and non-neural cells in cultures [3,5,6,7]. In none of the experimental systems examined so far has ethanol been found to enhance DNA synthesis.

In NIH 3T3 fibroblasts, ethanol has been shown to induce extensive hydrolysis of PtdEtn [8] in the presence of an activator of PKC. This prompted us to examine the effects of ethanol on DNA synthesis in these cells. Unexpectedly, while ethanol had no major effects on DNA synthesis induced by PDGF, FGF or lyso-PtdOH, it significantly enhanced the mitogenic effects of both insulin and IGF-1.

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Abbreviations used: IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; lyso-PtdOH, lysophosphatidic acid; PtdEtn, phosphatidylethanolamine; PI3K, phosphatidylinositol-3-kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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EXPERIMENTAL PROCEDURES

Materials and Methods: Insulin, IGF-1, PDGF-BB (human, recombinant) and basic FGF were bought from Boehringer Mannheim; wortmannin, lyso-PtdOH, PMA and Dowex-50W(H^+ form) were from Sigma; ethanol was from Baker and was redistilled prior to use; [methyl-^{3}H]thymide (85 Ci/mmol) was purchased from Amersham; and tissue-culture reagents were bought from GIBCO BRL.

Cell Culture: NIH 3T3 clone-7 fibroblasts were kindly provided by Dr. Douglas R. Lowy (National Cancer Institute, NIH, Bethesda, MD). Fibroblasts were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, penicillin-streptomycin (50 U/ml and 50 µg/ml, respectively), and glutamine (2 mM).

Labeling of Cellular DNA with [^{3}H]Thymidine: NIH 3T3 fibroblasts were first grown in 12-well tissue culture dishes to about 35-40% confluency in the presence of 10% serum. This was followed by incubation of fibroblasts in serum-free medium for 24 h, and then by treatments with the respective growth factors in the absence or presence of ethanol for 16 h. Finally, incubations were continued in the presence of [methyl-^{3}H]thymidine for 60 min. Fibroblasts were washed twice with phosphate-buffered saline and then four times with 5% trichloroacetic acid. The acid-insoluble material was redissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated ^{3}H activity in a liquid scintillation counter.

RESULTS AND DISCUSSION

Treatment of serum-starved NIH 3T3 fibroblasts with ethanol (10-150 mM) alone for 16 h failed to alter the low level of labeling of cellular DNA with [^{3}H]thymidine (Fig. 1). In these fibroblasts, 100 nM insulin, 500 nM insulin (maximally effective concentration) and 100 nM IGF-1 (about half-maximally effective concentration) enhanced DNA synthesis 9.1-, 12.1- and 7.7-fold, respectively. While 10 mM ethanol had no significant effects, 50 mM ethanol enhanced the stimulatory effects of growth factors on DNA synthesis 2- to 2.8-fold (Fig. 1). Further increase of ethanol concentration to 100 mM did not yield greater potentiating effects, while 150 mM ethanol had less than maximal effects (Fig. 1). In additional experiments we also determined the effects of ethanol on DNA synthesis in the presence of different concentrations of IGF-1. In the presence of 500 nM IGF-1 (maximally effective concentration), 50 mM ethanol enhanced DNA synthesis 2.2-fold (data not shown). We should note here that under the conditions used, there is some loss of ethanol from the incubation medium by the end of the 16 h incubation period; thus, the maximally effective concentration of ethanol might be less than 50 mM.

In NIH 3T3 fibroblasts, maximally effective concentrations of FGF, PDGF, and lyso-PtdOH were significantly more potent than insulin or IGF-1 in inducing DNA synthesis. As shown in Fig. 2, the effects of these growth factors were not significantly altered by 100 mM ethanol (Fig. 2).

In NIH 3T3 fibroblasts, ethanol was shown to increase phospholipase C-mediated hydrolysis of PtdEtn in the presence of a protein kinase C activator, such as PMA [8]. To test

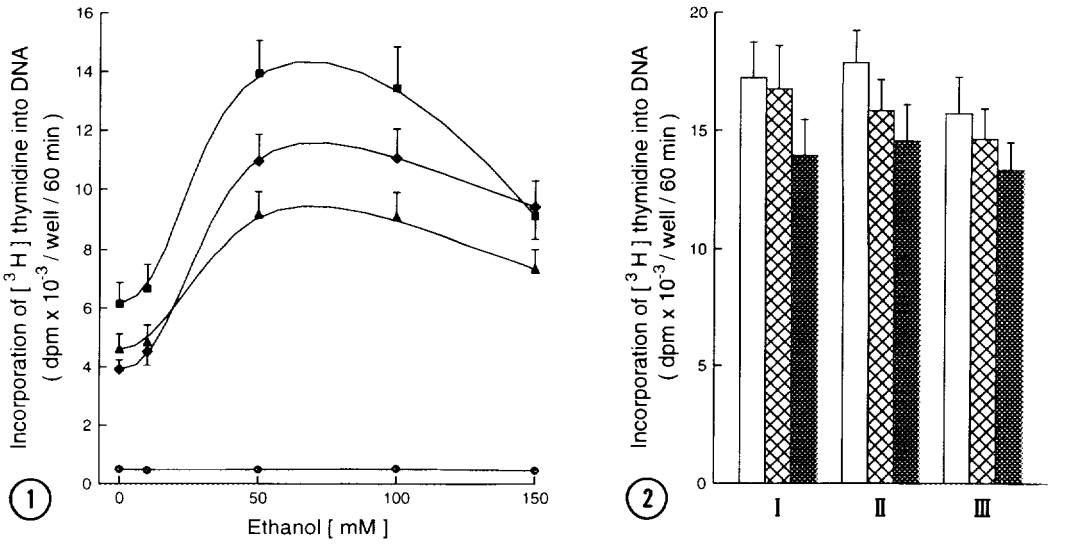

Figure 1. Concentration-dependent effects of ethanol on insulin- and IGF-1-induced DNA synthesis in NIH 3T3 fibroblasts.
Serum-starved (24 h) fibroblasts were treated with various concentrations (0-150 mM) of ethanol for 16 h in the absence (●) or presence of 100 nM insulin (▲), 500 nM insulin (■), or 100 nM IGF-1 (◆), followed by incubation of fibroblasts for 1 h in the presence of [^{3}H]thymidine. Each point represents the mean ± S.E. of three experiments performed with the same passage of cells. Similar results were obtained in six other experiments.

Figure 2. Combined effects of ethanol and growth factors on DNA synthesis in NIH 3T3 fibroblasts.
Serum-starved fibroblasts were treated for 16 h with 50 ng/ml of FGF (I), 50 ng/ml of PDGF (II), or 50 μg/ml of lyso-PtdOH (III) in the absence (□) or presence of 50 mM ethanol (▨) or 100 mM ethanol (■), followed by incubations in the presence of [^{3}H]thymidine for 1 h. Data represent the mean ± S.E. of three experiments performed with the same passage of cells. Similar results were obtained in three other experiments.

whether formation of ethanolamine phosphate and/or 1,2-diacylglycerol might play a role in the co-mitogenic effects of ethanol, next we examined the combined effects of PMA and ethanol on insulin-induced DNA synthesis. As shown in Fig. 3, PMA failed to significantly modify the effects of insulin and ethanol on DNA synthesis, which argues against the role of PtdEtn hydrolysis in the mediation of ethanol effects. In further support of this conclusion, neither insulin nor ethanol, alone or in combination, were found to exert significant stimulatory effects on phospholipase D- or phospholipase C-mediated hydrolysis of PtdEtn (data not shown).

The cellular actions of insulin in many cases involve PI3K [9-12]. In agreement with a possible role of PI3K in mediating the mitogenic effects of insulin in fibroblasts, wortmannin, an inhibitor of PI3K [13], inhibited the effects of insulin on DNA synthesis both in the absence and presence of ethanol (Fig. 3).

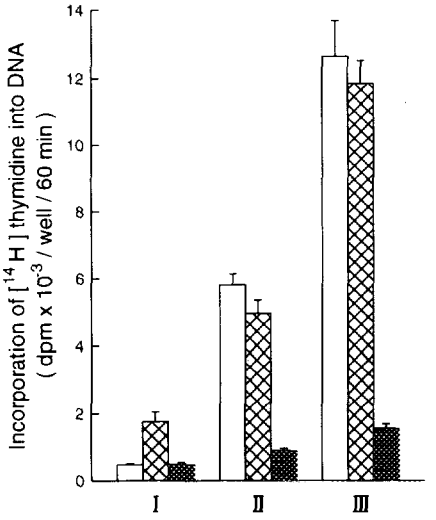

Figure 3. Effects of PMA and wortmannin on DNA synthesis induced by insulin and ethanol.
Serum-starved fibroblasts were incubated for 16 h in the absence (I) or presence of 500 nM insulin (II) or insulin plus 100 mM ethanol (III). During this treatment period, the incubation medium either contained no other additions (□), or contained 100 nM PMA (▨) or 100 nM wortmannin (■). Subsequent incubations in the presence of [^{3}H]thymidine were for 1 h. Data represent the mean ± S.E. of six experiments performed with the same passage of cells.

In summary, these data show for the first time that in a cultured cell line ethanol can enhance the stimulatory effects of specific growth factors on DNA synthesis. Thus, even if ethanol-induced growth inhibition is a widespread phenomenon, it is not universal.

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