The possible mechanism of synergistic effects of ethanol, zinc and insulin on DNA synthesis in NIH 3T3 fibroblasts

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Abstract In serum-starved NIH 3T3 fibroblast cultures, zinc (15–40 μM) enhanced both the individual and combined stimulatory effects of insulin and ethanol (EtOH) on DNA synthesis. Zinc, but not EtOH, also promoted the stimulatory effects of insulin on activating phosphorylation of p42/p44 mitogen-activated protein (MAP) kinases. In the presence of zinc, insulin induced premature expression of cyclin E during early G1 phase; EtOH partially restored the normal timing (late G1 phase) of cyclin E expression. The results suggest that zinc and EtOH promote insulin-induced DNA synthesis by different mechanisms; while zinc acts by enhancing the effects of insulin on MAP kinase activation, EtOH may act by ensuring timely zinc-dependent insulin-induced expression of cyclin E.

Key words: Ethanol; Zinc; Insulin; DNA synthesis; MAP kinase; Cyclin

1. Introduction

Although zinc is an important micronutrient which, in addition to other biological effects, is required for normal growth [1–3], relatively little is known about the possible interactions between zinc and growth factors in regulating mitogenesis. Earlier we found that in various cell lines, including NIH 3T3 fibroblasts, zinc was able to enhance insulin-induced DNA synthesis if ethanol (EtOH) was also present [4,5]. This suggested that the insulin-dependent growth regulatory mechanism may be a target for the actions of zinc.

Heavy alcohol consumption promotes development of certain cancers induced by carcinogens [6–10]. As suggested by others [11], these alcohol effects may be mediated, at least in part, by increased mitogenesis such as occurring in the simultaneous presence of EtOH, insulin and zinc [4,5]. Accordingly, the actual zinc status may influence the effect of EtOH, and possibly some growth factors, on carcinogenesis. In view of these possibilities, it is of interest to determine the signal transduction mechanism(s) mediating the effects of zinc, insulin and EtOH on mitogenesis. In this regard, in an earlier work we found that EtOH can enhance the stimulatory effect of insulin on p70 S6 kinase activity [5]. However, the promoting effect of EtOH was relatively small suggesting that there may be another mechanism involved in the mediation of EtOH effects on DNA synthesis.

Considering that activation of p42/p44 mitogen-activated protein (MAP) kinases is thought to be required for fibroblast growth [12], and that timely expression of cyclins, the activating partners of cyclin-dependent kinases (Cdk), are important for cell cycle progression [13], in this work we concentrated our efforts to determine the effects of zinc, insulin and EtOH on MAP kinase activation and cyclin expression. The results obtained suggest that zinc and EtOH are capable of enhancing the mitogenic effect of insulin by different mechanisms. Thus, while zinc most probably acts by promoting the effect of insulin on MAP kinase activation, EtOH is likely to act by shifting zinc-plus-insulin-induced expression of cyclin E from an early to a later phase of the G1 cell cycle period.

2. Materials and methods

2.1. Materials

Spectroscopic grade EtOH was purchased from Aldrich and was redistilled prior to use to remove possible traces of benzene (in [4], this EtOH preparation is referred to as preparation '5'); the first ~35% of the distillate was discarded. PD 98059 was bought from Calbiochem; zinc chloride was from Sigma; insulin was purchased from Boehringer Mannheim; the p42/p44 MAPK Western blot kit, including activating phosphorylation site (tyrosine-204)-specific antibody, was bought from New England Biolabs; rabbit polyclonal antibodies against cyclins A, D1, and E, as well as peroxidase-conjugated goat anti-rabbit IgG and protein A-agarose were bought from Santa Cruz Biotechnology; [*methyl*-^{3}H]thymidine (500 mCi/mmol) was purchased from Dupont NEN; and tissue culture reagents, including fetal calf serum, were from GIBCO-BRL.

2.2. Cell culture

NIH 3T3 clone-7 fibroblasts (American Type Culture Collection) were cultured in 10% fetal calf serum-containing Dulbecco's modified Eagle's medium as in [4].

2.3. Determination of DNA synthesis

Fibroblasts were grown in 12-well tissue culture plates to about 40% confluency in the presence of 10% serum, washed, incubated in serum-free medium for 24 h, and then washed again 3 h prior to treatments. The cells (70–80% confluent) were first treated (in serum-free medium; incubation volume, 0.75 ml) with 40 μM zinc chloride (dissolved in medium) for 10 min or 3 h, as indicated, and then with EtOH and insulin for 18 h. [*Methyl*-^{3}H]thymidine (1 μCi/well) was added for the last 60 min of treatment. In preliminary experiments we determined that in each case maximal incorporation of thymidine into DNA occurs between 17–18 h of treatment, and that none of these agents, alone or in combination, increases cellular uptake of thymidine during the first 15 h of treatment. At the conclusion of treatments, 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 ^{3}H activity in a liquid scintillation counter. The cell numbers in ~80% confluent cultures were in the range of $1.5–1.7\times10^5$/well.

2.4. Western blot analysis of phosphorylation of p42/p44 MAP kinases

Serum-starved (24 h) NIH 3T3 cells, grown in 35 mm diameter dishes to 70–80% confluency, were first incubated with 40 μM zinc

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Abbreviations: MAP, mitogen-activated protein; EtOH, ethanol; Cdk, cyclin-dependent kinase; Rb, retinoblastoma

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for 2 min, and then treated with 60 mM EtOH and/or insulin for 10 min, 1 h or 6 h. Samples for immunoblot analysis were prepared as indicated earlier [14,15]. Phosphospecific MAP kinase antibody was used to detect the phosphorylated (activated) forms of p42/p44 MAP kinases. The Western immunoblotting protocol was performed according to the instructions provided by the manufacturer. The relative changes in the intensity of protein phosphorylation was determined by Storm 840 phosphoimager (Molecular Dynamics).

2.5. Western blot analysis of cyclins

Serum-starved NIH 3T3 fibroblasts were treated for 6 or 12 h with zinc, insulin and/or EtOH as described in Fig. 4, then samples were prepared and subjected to Western blot analysis of cyclins as in [16], and the changes in cyclin expression were quantified as above.

3. Results

3.1. Zinc enhances insulin- and EtOH-stimulated DNA synthesis

In the absence of zinc, treatment of serum-starved NIH 3T3 fibroblasts with insulin resulted in only a slight increase in DNA synthesis, while 60 mM EtOH alone was without effects (Fig. 1A). Pretreatment of fibroblasts with 40 μM zinc for 3 h (Fig. 1A) or 10 min (Fig. 1B) prior to EtOH/insulin treatments similarly enhanced both the individual and combined effects of insulin and EtOH. The concentration of EtOH (60 mM) used was nearly optimal while that of insulin (500 nM) was optimal for these effects [4,5]. The combined treatments with these three agents increased DNA content about 1.7-fold as determined by ethidium bromide (not shown), indicating that increased [^{3}H]thymidine incorporation into DNA (Fig. 1) was indeed due to increased DNA synthesis. This conclusion is further supported by additional findings that zinc, insulin and EtOH had detectable effects on [^{3}H]thymidine incorporation into DNA only after an about 16 h incubation period, i.e. when cells would be expected to enter the DNA synthesizing S cell cycle period.

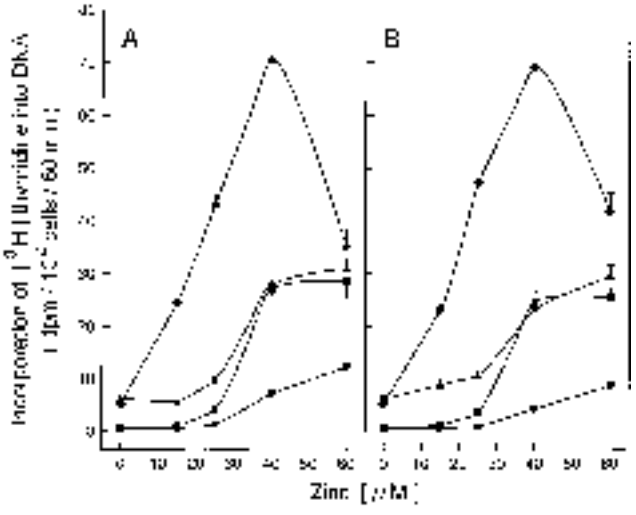

Fig. 1. Combined effects of zinc, insulin and EtOH on DNA synthesis in NIH 3T3 fibroblasts. Serum-starved (24 h) NIH 3T3 fibroblasts were incubated first with 0–40 μM zinc for 3 h (A) or 10 min (B), as indicated, followed by incubations for 18 h in the absence (●) or presence of 500 nM insulin (▲), 60 mM EtOH (■) or insulin plus EtOH (♦); [^{3}H]thymidine was added for the last 60 min of the treatment. Each point represents the mean ± S.D. of six incubations in a representative experiment. Similar results were obtained in two other experiments each performed in triplicate.

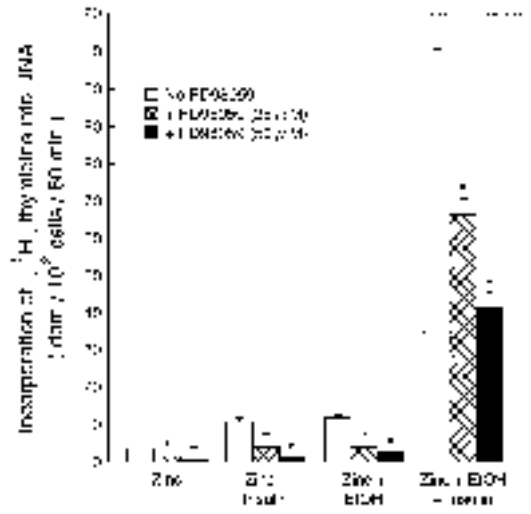

Fig. 2. Inhibitory effects of PD 98059 on DNA synthesis induced by zinc, insulin and EtOH in NIH 3T3 fibroblasts. Serum-starved NIH 3T3 cells were first treated with 40 μM zinc for 3 h, followed by incubations for 18 h in the absence or presence of 500 nM insulin and/or 60 mM EtOH as indicated; PD 98059 was either absent (□) or present at 25 μM (▨) or 50 μM (■) concentrations. The data are the means ± S.D. of six incubations in a single experiment. Similar inhibitory effects were obtained in two other experiments each performed in triplicate. *Significantly ($P < 0.01$) different from the corresponding value in the absence of inhibitor (Student's *t*-test).

Lower (15–25 μM) concentrations of zinc significantly promoted DNA synthesis only if insulin and EtOH were simultaneously present. Again, the potentiating effects of lower concentrations of zinc were about the same when zinc was added 3 h (Fig. 1A) or only 10 min (Fig. 1B) prior to EtOH and insulin. Since cellular uptake of zinc is known to be a slow process, these data strongly suggest that the observed effects of zinc on DNA synthesis may not require its incorporation into cells.

The MEK1 inhibitor PD 98059 [17], used at 25–50 μM concentrations (which have been commonly used in cell cultures to achieve optimal effects), inhibited both the single and combined effects of zinc, insulin and EtOH on DNA synthesis (Fig. 2). This suggested that MAP kinase activation was im-

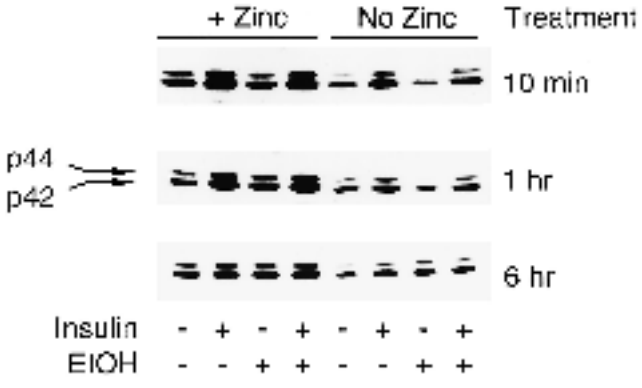

Fig. 3. Western blot analysis of the effects of zinc, insulin and EtOH on the phosphorylation of p42/p44 MAP kinases. Serum-starved NIH 3T3 cells were first treated with 40 μM zinc for 2 min, followed by incubations for 10 min, 1 h, or 6 h in the absence or presence of 500 nM insulin and/or 60 mM EtOH as indicated. This experiment was repeated once with similar results.

portant in each case, although not each agent necessarily activated MAP kinases. To clarify the contribution of MAP kinases to the mitogenic effects of zinc, insulin and EtOH, we next determined the ability of these agents to induce activating phosphorylations of p42/p44 MAP kinases.

3.2. Effects of zinc, insulin and EtOH on activating phosphorylations of MAP kinases

In the absence of zinc, insulin had only small stimulatory effects on MAP kinase phosphorylation at the tyrosine site which is known to result in enzyme activation [18] (Fig. 3). Zinc (40 μM) alone enhanced phosphorylation of MAP kinases and, in addition, it clearly promoted the stimulatory effects of insulin particularly after 10 and 60 min treatments (Fig. 3). In contrast, EtOH (60 mM) alone or in combination with zinc and/or insulin had no effects on MAP kinase phosphorylation (Fig. 3).

3.3. Effects of zinc, insulin and EtOH on the expression of cyclins

Both in the absence of zinc (not shown) and in its presence (Fig. 4), insulin similarly enhanced expression of cyclin A after 12 h, but not 6 h, treatment. It should be added that maximum insulin-induced expression of cyclin A occurred after about 16 h of starting the treatment with insulin (not shown), indicating a normal expression pattern for this cyclin [19]. EtOH alone (not shown) or in the presence of insulin and zinc had no effects on cyclin A expression (Fig. 4).

In the presence of zinc, expression of cyclin D1 was enhanced by insulin during the first 6 h of treatment (i.e. during early G1 phase), as expected [19], followed by decreased expression between 6–12 h of treatment. Insulin had very similar effects on cyclin D1 expression in the absence of zinc (not shown). Again, zinc and EtOH did not alter cyclin D1 expression (Fig. 4).

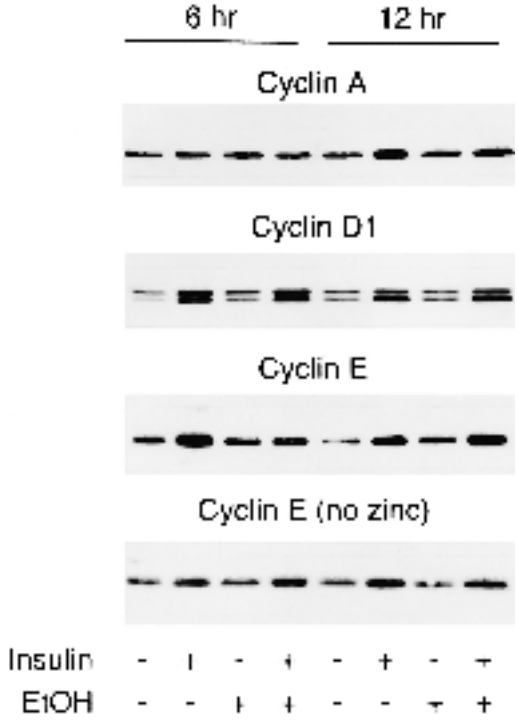

Fig. 4. Western blot analysis of the effects of zinc, insulin and EtOH on the expression of cyclins. Serum-starved NIH 3T3 cells were first treated with 40 μM zinc for 2 min, unless indicated otherwise, followed by incubations for 6 or 12 h in the absence or presence of 500 nM insulin and/or 60 mM EtOH, as indicated. This experiment was repeated twice with similar results.

Unexpectedly, in the presence of zinc, but not in its absence, insulin induced strong expression of cyclin E during the first 6 h of treatment (Fig. 4). Co-treatment with EtOH clearly shifted zinc-plus-insulin-induced expression of cyclin E from the 0–6 h treatment period to the 6–12 h period (Fig. 4). Accordingly, in the presence of EtOH, zinc plus insulin induced the synthesis of about 2.1-fold more cyclin E during the 6–12 h treatment period compared to the first 6 h, while the same treatments performed in the absence of EtOH produced about 1.9-fold more cyclin E during the first 6 h (Fig. 4).

4. Discussion

In NIH 3T3 fibroblasts, insulin alone has proved to be a weak inducer of both DNA synthesis and MAP kinase activation. However, we have found that in these cells zinc can enhance the stimulatory effects of insulin on both the phosphorylation/activation of p42/p44 MAP kinases and DNA synthesis. Since activation of MAP kinases is important for fibroblast proliferation [12], it is reasonable to suggest that there is a causal relationship between the above potentiating effects of zinc.

Zinc had similar potentiating effects when used 3 h or 10 min prior to other treatments. Since cellular uptake of zinc is slow, these data suggest that zinc may exert its effects without entering the cells. In this context, it was recently suggested that in hepatocytes, mobilization of intracellular calcium by added zinc is mediated by a putative cell membrane ion receptor protein [20]. The possible role of a metal ion receptor in the mediation of zinc effects on DNA synthesis and MAP kinase activation is presently under study in this laboratory.

Interestingly, although EtOH had no effects on zinc-plus-insulin-induced MAP kinase activation, it still was able to greatly enhance the combined stimulatory effects of zinc and insulin on DNA synthesis. Detailed examination of expression of cyclins revealed that insulin can greatly enhance cyclin E expression only in the presence of zinc. However, while we previously observed fibroblast growth factor-induced cyclin E expression during late G1 phase [16], when it should normally appear (see, for example, ref. [13]), in the presence of zinc and insulin cyclin E appeared during the first 6 h of treatment, i.e. during the early G1 period. EtOH clearly shifted the bulk of cyclin E expression from an earlier to a later phase of the G1 cell cycle period. In contrast to cyclin E expression, the stimulatory effects of insulin on the expression of cyclins D1 and A were not affected by zinc and EtOH, and these cyclins appeared in a timely fashion as described for other stimulators of the cell cycle [13].

It appears that execution of a productive cell cycle requires that expression of various cyclins remains on schedule. This is because the various cyclins, together with the corresponding Cdks, have specific biological functions at a given time of the cell cycle. For example, Cdk4-cyclin D complexes induce the release of E2F transcription factor from the retinoblastoma (pRb)-E2F inhibitory complex by phosphorylating specific sites on pRb. Released E2F then induces transcription of cy-

clin E, among other proteins, leading to further phosphorylation of pRb by the Cdk2-cyclin E complex as well as to a large increase in E2F-dependent transcription and the onset of S phase [13]. Accordingly, it is likely that the induction of early expression of cyclin E by zinc and insulin disturbs the normal sequence of cell cycle to an extent that their large effects on MAP kinase activation cannot translate to a similarly large effect on DNA synthesis. Based on these considerations, it is conceivable that the ability of EtOH to shift the bulk of zinc-plus-insulin-induced cyclin E expression from an earlier to a later G1 phase significantly contributes to its zinc-dependent potentiating effects on insulin-induced DNA synthesis.

In summary, we have found that while zinc can enhance insulin-induced DNA synthesis by a MAP kinase-dependent mechanism, zinc and insulin also induce abnormal expression of cyclin E which appears to limit the extent of DNA synthesis. In the presence of EtOH, zinc-plus-insulin-induced expression of cyclin E becomes more normal, probably accounting for the zinc-dependent potentiating effect of EtOH on insulin-induced DNA synthesis. Based on these observations, it appears worthwhile to further examine the possible relationship between the zinc- and insulin-dependent effects of EtOH on DNA synthesis and carcinogenesis.

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