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Inhibition of insulin-like growth factor-I mitogenic action by zinc chelation is associated with a decreased mitogen-activated protein kinase activation in RAT-1 fibroblasts

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Abstract The mechanisms responsible for the resistance to the anabolic actions of IGF-I induced by zinc deficiency are not understood. We showed that zinc chelation by DTPA (diethylenetriaminepenta-acetic acid) inhibits [^{3}H]thymidine incorporation stimulated by IGF-I in Rat-1 fibroblasts. This inhibition was specific of zinc chelation since it was prevented by the addition of zinc to DTPA. The stimulation of MAPK, which is crucial for the [^{3}H]thymidine incorporation induced by IGF-I in Rat-1 cells, was partially blunted by DTPA. Therefore, the inhibition of the mitogenic action of IGF-I in Rat-1 fibroblasts by DTPA is potentially caused by decreased MAPK activation by IGF-I.

Key words: Zinc chelation; Insulin-like growth factor-I; Diethylenetriaminepenta-acetic acid; Mitogen-activated protein kinase

1. Introduction

Insulin-like growth factor-I (IGF-I) is an important growth factor which mediates many of the growth-promoting effects of growth hormone (GH). In animals and in humans, dietary zinc deficiency causes growth retardation associated with reduced circulating IGF-I concentrations [1–4]. IGF-I administration to zinc-deprived rats fails, however, to restore the growth despite the normalization of plasma IGF-I levels [5]. These observations suggest that zinc deficiency induces a state of resistance to the growth-promoting actions of IGF-I.

To investigate the mechanisms by which zinc deficiency could inhibit the growth-promoting actions of IGF-I, we studied the effects of zinc chelation on the mitogenic and anabolic actions of IGF-I in Rat-1 fibroblasts. Indeed, IGF-I is known to stimulate the proliferation and amino acid transport in these cells [6,7]. Because the mitogen-activated protein kinase (MAPK) signalling cascade is important for the mitogenic action of IGF-I [8], we also investigated whether inhibition of IGF-I mitogenic action by zinc chelation was associated with a decreased activation of the MAPK isoforms ERK1 and ERK2.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics and glutamine were obtained from Life Technologies (Merelbeke, Belgium). IGF-I was a gift from Pharmacia-Upjohn (Uppsala, Sweden). DTPA (diethylenetriaminepenta-acetic acid) and $ZnSO_4$ were purchased from Sigma Chemical (St Louis, MO, USA). [^{3}H]thymidine was obtained from Amersham (Buckinghamshire, UK, specific activity(SA): 25 Ci/mmol) and [methyl-^{3}H]AIB was obtained from ICN Biomedicals (Asse-Relegem, Belgium, SA: 1-10 Ci/mmol).

2.2. Cell culture

Rat-1 fibroblasts, kindly provided by Dr P. Courtoy (ICP, Brussels, Belgium) were cultured in DMEM, as described previously [9].

2.3. [^{3}H]thymidine incorporation assay

Cells were seeded (10^4 cells/well) in 96 well tissue culture plates (NUNC, Naperville, IL, USA) in DMEM with 10% FBS. 24 h after plating, cells were exposed for different times to IGF-I in serum-free medium with 0.1% BSA (control group). In order to test the consequences of zinc deficiency on the mitogenic action of IGF-I, 40 μM DTPA (DTPA group) or 40 μM DTPA with 40 μM $ZnSO_4$ (DTPA+$ZnSO_4$ group) were added to the culture medium. 5 h prior to the cell harvest, [^{3}H]thymidine (1 μCi) was added. Cells were washed, detached in trypsin-EDTA and harvested onto glass-fiber filters using a cell harvester Filtermate 196 (Packard, Meriden, CT, USA). The counts per well of triplicate or sixplicate determinations were determined using a microplate β-scintillation counter (Packard), averaged and expressed as the percentage of total counts in untreated cells

2.4. [Methyl-^{3}H]AIB uptake

Cells were seeded (6×10^4 cells/well) in 24 well tissue culture plates (NUNC) in DMEM with 10% FBS for 48 h with a change of the medium after 24 h. IGF-I stimulation of the [methyl-^{3}H]AIB uptake was performed as described previously [10]. IGF-I was added for 6 h to duplicate wells in HBSS (control group) or in the presence of 40 μM DTPA, or 40 μM DTPA and 40 μM $ZnSO_4$. The counts per well of duplicate determinations were averaged and expressed as the percentage of counts in untreated cells.

2.5. MAPK assay

Cells were seeded (3×10^5 cells/well) in six well plates in DMEM with 10% FBS for 48 h. Cells were then washed and incubated in triplicate in serum-free medium or in the presence of 40 μM DTPA or 40μM DTPA plus 40μM $ZnSO_4$ for 24 h. IGF-I was then added for 2, 5, 10 or 15 min. The IGF-I stimulation was stopped by chilling plates on ice and removing the medium. Cells were washed, scraped in MAPK lysis buffer [11] and the lysates from triplicate wells were pooled. Cell lysates were resolved by SDS-PAGE on 10% gels and then transferred to Hybond C nitrocellulose (Amersham Life Science, Arlington Heights, IL, USA). Western blotting of catalytically active MAPK was performed using an anti-phospho-MAPK antibody at 1 μg/ml that recognizes dual-phosphorylated ERK1 and ERK2

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Abbreviations: MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ERK, extracellular regulated kinase; AIB, aminoisobutyric acid

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(New England Biolabs, Beverly, MA, USA). The blots were stripped and probed with anti-rat MAPK R2 (ERK1-CT) antibody (Upstate Biotechnology, Lake Placid, NY, USA) at 1 μg/ml to determine the total ERK1 and ERK2 contents. Specific binding was visualized using enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA) followed by scanning densitometry. Data are expressed as the ratio of phosphorylated MAPK/total MAPK.

2.6. Statistical analysis

Data were analyzed according to two-way analysis of variance. Multiple comparisons were performed using the pairwise Bonferroni multiple comparison test. Data from Fig. 1 were transformed to their logarithm before the statistical analyses. On graphs, data are expressed as the mean ± S.E.M. Differences were considered significant when $P < 0.05$.

3. Results

3.1. The effect of zinc chelation on the mitogenic action of IGF-I

We first examined whether zinc chelation could inhibit the IGF-I-stimulated DNA synthesis in Rat-1 fibroblasts. As shown in Fig. 1, IGF-I stimulated dose-dependently the [^{3}H]thymidine incorporation in these cells. The maximal stimulation (3-fold) was observed at 10 nM IGF-I while the half-maximal stimulation occurred at 0.4 nM (3 ng/ml). To mimic a state of zinc deficiency, we incubated the cells in the presence of the specific zinc chelator, DTPA. DTPA decreased the basal thymidine incorporation by 66 ± 8% (DTPA w/o IGF-I versus control w/o IGF-I, $P < 0.01$, $n = 3$, Fig. 1). DTPA also markedly inhibited the maximal IGF-I stimulation of

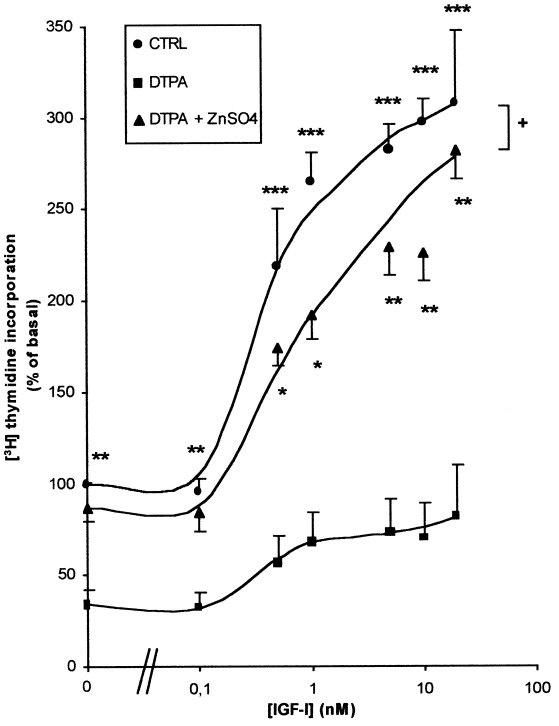

Fig. 1. The effect of zinc chelation by DTPA on the IGF-I-stimulated [^{3}H]thymidine incorporation in Rat-1 fibroblasts. Results are expressed as percentage of the values observed in untreated cells (control w/o IGF-I: 8367 ± 1298 cpm/well). Data are the mean ± S.E.M. of three separate experiments. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ versus the corresponding DTPA group at each IGF-I concentration. $^+$, $P < 0.05$ versus the control group at the different IGF-I concentrations.

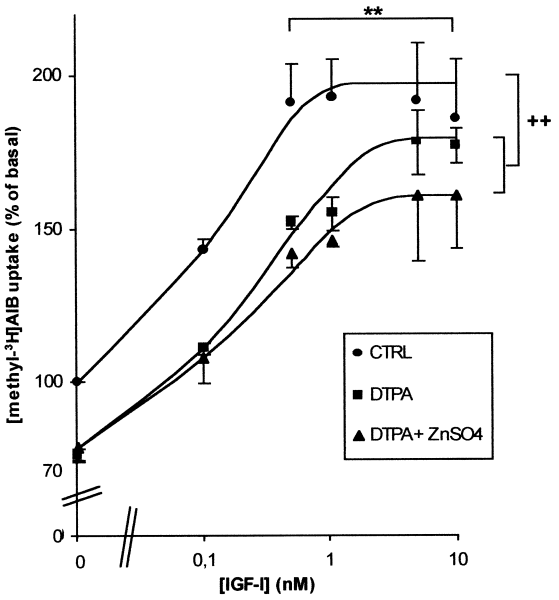

Fig. 2. The effect of zinc chelation by DTPA on the IGF-I-stimulated [methyl-^{3}H]AIB uptake in Rat-1 fibroblasts. Results are expressed as percentage of the values observed in untreated cells (control w/o IGF-I: 3286 ± 495 cpm/well). Data are the mean ± S.E.M. of three separate experiments. **, $P < 0.01$ versus the control group w/o IGF-I and $^{++}$ versus the control group at the different IGF-I concentrations.

[^{3}H]thymidine incorporation (by 75% at 10 nM IGF-I, $P < 0.001$). The inhibition of IGF-I-stimulated [^{3}H]thymidine incorporation by 40 μM DTPA was almost completely prevented by the addition of 40 μM $ZnSO_4$ (20 nM IGF-I, 3-fold stimulation versus 20 nM IGF-I+DTPA+$ZnSO_4$, 2.8-fold stimulation: not significant (N.S.) (Fig. 1).

The mitogenic action of IGF-I in Rat-1 cells was also confirmed by the increase of the total DNA content (+22% at 5–10 nM IGF-I, $P < 0.05$, $n = 3$) (data not shown). Similarly to the [^{3}H]thymidine incorporation, the increase in the DNA content induced by IGF-I was also inhibited by DTPA and restored to normal by the addition of $ZnSO_4$.

The kinetic analysis of the inhibition of the IGF-I mitogenic action by DTPA (data not shown) indicated that DTPA inhibited time-dependently the IGF-I-induced thymidine incorporation (by 50% after 12 h, by 90% after 18 h and by 97% after 24 h, $P < 0.001$, $n = 2$). Together, these data demonstrated that zinc chelation by DTPA blocked the IGF-I-stimulated DNA synthesis.

3.2. The effect of zinc chelation on IGF-I-stimulated [methyl-^{3}H]AIB uptake

We questioned whether DTPA might inhibit other biological actions of IGF-I such as the amino acid uptake in Rat-1 fibroblasts. While DTPA, alone or together with $ZnSO_4$, decreased the basal [methyl-^{3}H]AIB uptake by respectively 24 ± 4% and 21 ± 4% (N.S., $n = 3$), the stimulation by IGF-I of the [methyl-^{3}H]AIB uptake was not impaired by DTPA or DTPA+$ZnSO_4$ (10 nM IGF-I stimulation by 1.9-fold, 10 nM IGF-I+DTPA by 1.8-fold, 10 nM IGF-I+DTPA+$ZnSO_4$ by 1.6-fold: N.S., $n = 2$) (Fig. 2). These data showed that zinc chelation by DTPA did not inhibit the amino acid uptake induced by IGF-I in Rat-1 fibroblasts.

3.3. The effect of zinc chelation on the IGF-I-stimulated MAPK activity

Given that the activation of the MAPK isoforms ERK1 and ERK2 is a common pathway used by IGF-I and other growth factors to stimulate mitogenesis [12], we examined the effect of zinc chelation on the MAPK activation by IGF-I. We first determined that this pathway is involved in the stimulation of [^{3}H]thymidine incorporation by IGF-I in Rat-1 fibroblasts. The addition of PD98059, a specific inhibitor of the MAPK kinase (MEK) activity [13,14], completely inhibited indeed the stimulation of the [^{3}H]thymidine incorporation by IGF-I (by 88% at 10 μM PD98059 and by 96% at 50 μM, $P < 0.001$) (Fig. 3A). In the absence of IGF-I, PD98059 also inhibited the basal [^{3}H]thymidine incorporation. By measuring dual-phosphorylated MAPK, we showed that IGF-I induced the MAPK activation in a time-dependent manner (maximum of 4.5-fold at 2 min, $P < 0.001$, $n = 2$) and we confirmed that the MAPK activation by IGF-I was prevented by pretreatment with the inhibitor PD98059 (by 57% at 10 μM PD98059, $P < 0.05$ and by 79% at 50 μM, $P < 0.01$) (Fig. 3B and C). In the absence of IGF-I, PD98059 also inhibited the basal MAPK phosphorylation. These results demonstrate that p42/p44 MAPKs are involved in the stimulation of thymidine incorporation by IGF-I in Rat-1 fibroblasts. We then examined the effect of zinc chelation on the MAPK activation by IGF-I. The IGF-I stimulation of MAPK was significantly blunted by DTPA, by 31% at 2 min and by 42% at 5 min ($P < 0.01$ versus IGF-I group, $n = 2$) (Fig. 4). The addition of $ZnSO_4$ concomitantly to DTPA almost completely

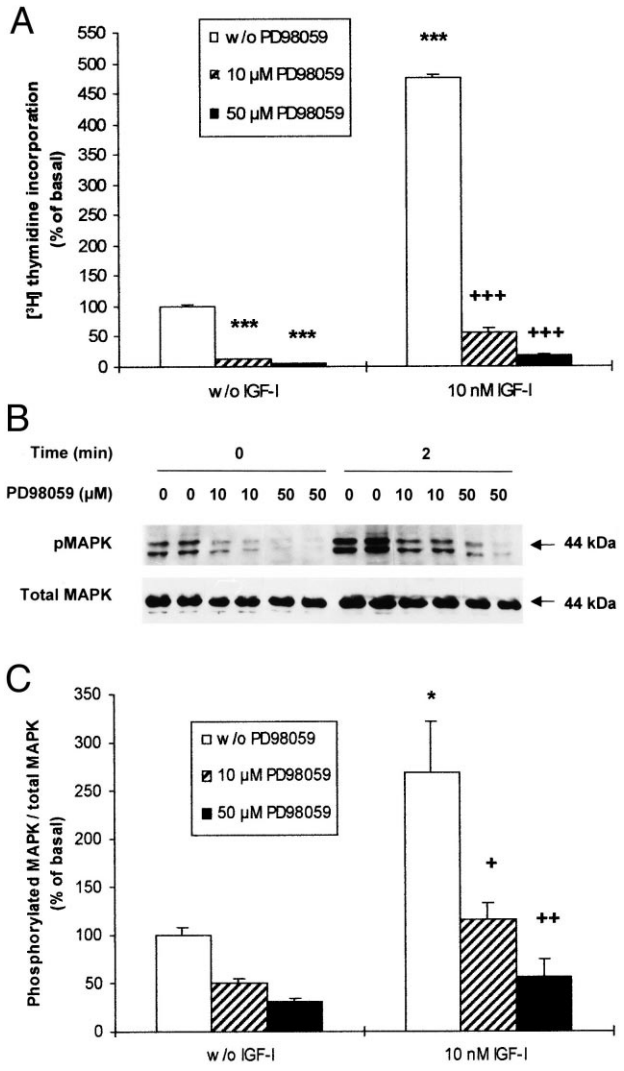

Fig. 3. The effect of PD98059 on the stimulation by IGF-I of the[^{3}H]thymidine incorporation and MAPK phosphorylation in Rat-1 fibroblasts. (A) [^{3}H]thymidine incorporation. Results are expressed as the percentage of the values observed in untreated cells (w/o IGF-I and w/o PD98059: 24646 ± 526 cpm/well). Data are the mean ± S.E.M. of sixplicates of one experiment. ***, $P < 0.001$ versus w/o IGF-I and w/o PD98059, $^{+++}$, $P < 0.001$ versus IGF-I w/o PD98059. (B) Western blot of active MAPK and total MAPK. (C) A densitometric analysis of the Western blot of active MAPK and total MAPK was used to determine the ratio of phosphorylated MAPK to total MAPK. Results are expressed as the percentage of the values observed in untreated cells (w/o IGF-I and w/o PD98059). Data are the mean ± S.E.M. of triplicates of one experiment. *, $P < 0.05$ versus w/o IGF-I and w/o PD98059; $^{+}$, $P < 0.05$ and $^{++}$, $P < 0.01$ versus IGF-I w/o PD98059.

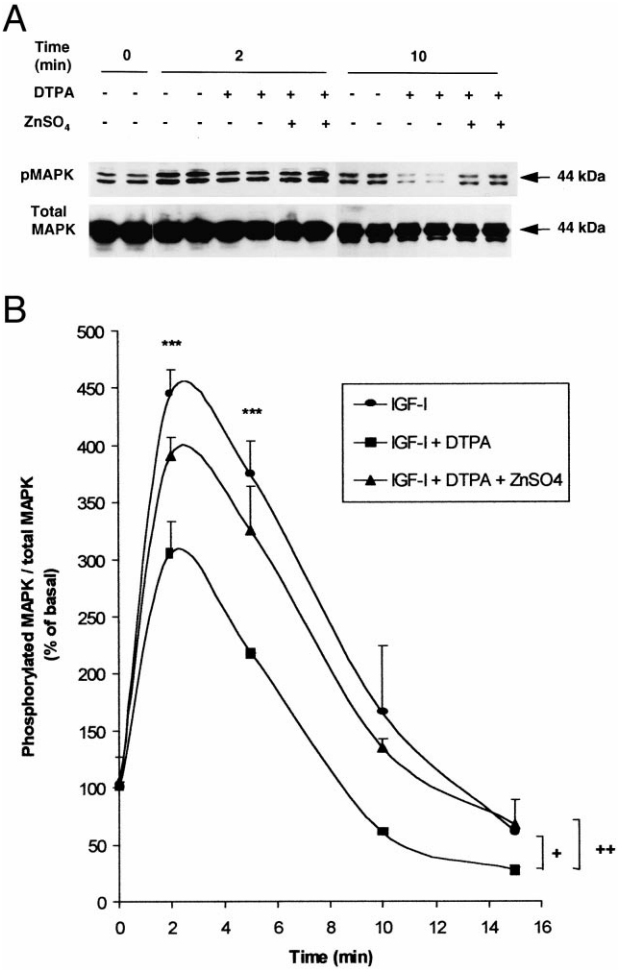

Fig. 4. Time course of the effect of zinc chelation by DTPA on the stimulation of the MAPK phophorylation by IGF-I in Rat-1 fibroblasts. (A) Representative Western blot of active MAPK and total MAPK. (B) Densitometric analysis of the Western blot was used to determine the ratio of phosphorylated MAPK to total MAPK. Results are expressed as the percentage of the values observed in untreated cells (w/o IGF-I at time 0). Data are the mean ± S.E.M. of two separate experiments. ***, $P < 0.001$ versus control 0′; $^{+}$, $P < 0.05$ between the groups IGF-I and IGF-I+DTPA+$ZnSO_4$, $^{++}$, $P < 0.01$ between the groups IGF-I and IGF-I+DTPA.

restored the levels of phosphorylated MAPK (88% of the control values at 2 and 5 min, IGF-I group versus IGF-I+DTPA+$ZnSO_4$ group, N.S., $n=2$) (Fig. 4). In the absence of IGF-I (time 0), DTPA alone did not modify the basal phosphorylation of MAPK (Fig. 4).

4. Discussion

We showed that zinc chelation by DTPA inhibits the mitogenic action of IGF-I in Rat-1 fibroblasts. This inhibition could be explained by a decrease in the MAPK activation, a crucial step in the transduction of the mitogenic signal initiated by IGF-I in these cells.

Our study showed that the zinc availability is critical for DNA synthesis both in basal conditions and in response to IGF-I. Furthermore, this inhibition of the DNA synthesis, as assessed by [^{3}H]thymidine incorporation, was confirmed by a decreased cellular DNA content. Therefore, the inhibition of the DNA synthesis by DTPA suggests that zinc is mandatory for the cell entry into the S phase of the cell cycle [15–19]. This conclusion is supported by data of Chesters et al. showing that zinc is required during the G1 to S phase transition [20].

The inhibition of the DNA synthesis by DTPA was specifically caused by zinc depletion because it was almost completely prevented by the addition of zinc to DTPA. Furthermore, the addition of Fe^{3+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+} and Ca^{2+} to DTPA did not restore the stimulation of thymidine incorporation by IGF-I or serum [16,21]. This suggests that it is the chelation of zinc per se and probably not the chelation of other divalent cations which was responsible for these effects. Moreover, these observations also suggest that it is unlikely that the observed effect of DTPA was due to toxic effects of the compound itself.

Although zinc chelation completely blocked the IGF-I stimulation of thymidine incorporation, it did not impair the stimulation of amino acid uptake, another biological action of IGF-I. This suggests that the bioavailability of IGF-I is not altered by zinc chelation and that the mechanism of inhibition of the IGF-I mitogenic action by zinc chelation is likely a post-receptor defect. Although DTPA slightly decreased the basal amino acid uptake, the decrease was not prevented by zinc addition, suggesting that this effect is not the consequence of zinc depletion and might be due to the chelation of other divalent cations important for the transport of amino acids.

To further elucidate the mechanisms by which zinc chelation inhibits the mitogenic action of IGF-I, we investigated the effect of zinc chelation on post-receptor signal transduction. It has been recently shown that the DTPA inhibition of IGF-I stimulation of the DNA synthesis in Swiss 3T3 fibroblasts is not mediated by a decrease in IGF-I receptor levels [21–22]. In our study, we determined whether the inhibition by DTPA of the IGF-I mitogenic action was associated with an alteration in the MAPK activation, an important signalling cascade utilized by several growth factors to mediate mitogenesis [12]. Using PD98059 to inhibit the activation of MAPK [13,14], we demonstrated that MAPK activation is crucial for the stimulation of [^{3}H]thymidine incorporation by IGF-I in Rat-1 fibroblasts, as in other cells [23]. Our data demonstrate for the first time that zinc chelation by DTPA decreased the MAPK activation by IGF-I and addition of zinc to DTPA mostly restored the MAPK activation. Our study therefore sustains the role of zinc in the activation of MAPK in response to growth factors, as suggested by Kiss et al. [19]. Taken together, these observations suggest that alterations in the MAPK activity might be partially responsible for the inhibition of the IGF-I-stimulated thymidine incorporation by DTPA. Indeed, in Swiss 3T3 cells, Dudley et al. have established a correlation between the inhibition of thymidine incorporation and the inhibition of stimulation of MAPK, using increased concentrations of PD98059 [14]. Our observation that PD98059 inhibits the MAPK activation by IGF-I to a lesser extent than thymidine incorporation (Fig. 3) suggests that a partial decrease of the MAPK activation might be sufficient to induce an almost complete inhibition of the thymidine incorporation stimulated by IGF-I. The fact that MAPK phosphorylation levels are not completely restored by the addition of zinc to DTPA might be explained by a non-specific chelation by DTPA of Mg^{2+}, a divalent cation necessary for the activity of kinases. Although the MAPK activation by IGF-I was decreased by DTPA, the basal levels of phosphorylated MAPK (i.e. in the absence of IGF-I) were not decreased by DTPA. This suggests that DTPA does not act on the MAPK pathway as PD98059 and that the MAPK inactivation is not responsible for the decreased basal DNA synthesis caused by DTPA. Other cellular alterations downstream of MAPK (i.e. thymidine kinase) [24,25] and induced by zinc chelation might explain the decline in basal thymidine incorporation.

In vivo, dietary zinc deficiency induces a growth retardation and an insensitivity to the action of IGF-I [5]. Our observation that zinc chelation specifically inhibits the mitogenic action of IGF-I suggests that the insensitivity to IGF-I induced by zinc deficiency in vivo might be directly due to a reduced zinc availability.

In conclusion, we showed that the loss of the mitogenic activity of IGF-I by zinc chelation in Rat-1 fibroblasts is associated with a decrease in the activation of MAPK. This is the first demonstration that depletion of a trace element specifically induces a failure in the mitogenic transduction signal initiated by a growth factor.

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