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Rapid Letter

Differential Modulation of MAP Kinases by Zinc Deficiency in IMR-32 Cells: Role of H_2O_2

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ABSTRACT

The influence of zinc deficiency on the modulation of the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) was studied. Using human IMR-32 cells as a model of neuronal cells, the role of oxidants on MAPKs and activator protein-1 (AP-1) activation in zinc deficiency was investigated, characterizing the participation of these events in the triggering of apoptosis. Relative to controls, cells incubated in media with low zinc concentrations showed increased cell oxidants and hydrogen peroxide (H_2O_2) release, increased JNK and p38 activation, high nuclear AP-1-DNA binding activity, and AP-1-dependent gene expression. Catalase addition to the media prevented the increase of cellular oxidants and inhibited JNK, p38, and AP-1 activation. Low levels of ERK1/2 phosphorylation were observed in the zinc-deficient cells in association with a reduction in cell proliferation. Catalase treatment did not prevent the above events nor the increased rate of apoptosis in the zinc-deficient cells. It is first demonstrated that a decrease in cellular zinc triggers H_2O_2-independent, as well as H_2O_2-dependent effects on MAPKs. Zinc deficiency-induced increases in cellular H_2O_2 can trigger the activation of JNK and p38, leading to AP-1 activation, events that are not involved in zinc deficiency-induced apoptosis. *Antioxid. Redox Signal.* 7, 1773–1782.

INTRODUCTION

Zinc deficiency is recognized by the World Health Organization as a leading cause of human morbidity and mortality (32). Given the numerous roles zinc has in normal cell metabolism, zinc deficiency-induced pathologies can arise through multiple mechanisms, including alterations in cell membrane stability, impaired microtubule polymerization, perturbations in the binding of transcription factors, hormones, and signaling molecules that involve zinc binding fingers, and oxidative stress (17).

An early consequence of zinc deficiency is an increased production of oxidant species, and subsequent cellular oxidative damage (13, 21, 22). Thus, an increased steady-state level of cellular oxidants could trigger select signaling cascades in zinc deficiency, such as the oxidant-responsive transcription factors activator protein-1 (AP-1) (22) and nuclear factor-κB (19).

AP-1 activity is induced by a wide variety of physiological stimuli and stress conditions that lead to the modulation of important cellular processes, including the decision of cells to proliferate, differentiate, survive, or die by apoptosis (26). Mitogen-activated protein kinases (MAPKs) participate in the regulation of gene expression in part through the downstream modulation of the transcription factor AP-1 (23). The MAPK signaling pathways primarily consist of three major subfamilies: c-Jun N-terminal kinase (JNK) and p38 subfamilies, which are mainly responsive to stress (29), and extracellular signal-regulated kinase (ERK) subfamilies, which are mostly

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triggered by mitogenic signals. JNK and p38 are sensitive to increases in the intracellular levels of oxidants, being activated by UV radiation and hydrogen peroxide (H_2O_2) (15, 16). p38 can activate AP-1 through the phosphorylation of activating transcription factor-2 (ATF-2), MEF2C, and ternary complex factors. The activation of JNK is followed by translocation to the nucleus where it phosphorylates c-Jun and ATF-2, leading to an increase in c-Jun transcriptional capacity and *c-jun* transcription. Although ERK1/2 are particularly responsive to mitogenic stimuli, they can also be stimulated by oxidants (2, 9).

In the present study, using IMR-32 neuroblastoma cells, the role of MAPKs in the activation of AP-1 associated with zinc deficiency was studied. The involvement of oxidants in the regulation of MAPKs and AP-1 transcriptional activity, and on the decision of cells to die by apoptosis, was also investigated.

MATERIALS AND METHODS

Materials

IMR-32 cells and c-Jun cDNA were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cell culture media and reagents, and LipofectAMINE™ 2000, were obtained from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.). The oligonucleotides containing the consensus sequences for AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3′) and octamer binding transcription factor-1 (OCT-1), the reagents for the electrophoretic mobility shift assay (EMSA), the CellTiter 96 Non Radioactive Cell Proliferation assay, the enzyme assay systems for the determination of luciferase and β-galactosidase activities, and the pSV-β-galactosidase control vector were obtained from Promega (Madison, WI, U.S.A.). The PathDetect AP-1 *cis*-reporting system was obtained from Stratagene (La Jolla, CA, U.S.A.). Stress-activated protein kinase (SAPK)/JNK assay kit and p38 MAPK, antibodies for ERK1/2, SAPK kinase-1 (SEK1), and phosphorylated p38 (p-p38) and SEK1 were from Cell Signaling Technology (Beverly, MA, U.S.A.). Antibodies for p53, phosphorylated ERK (p-ERK), p38, phosphorylated JNK (p-JNK), JNK, c-Fos, c-Jun, JunD, JunB, FosB, Fra-1, Fra-2, and β-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCDCDHF) and propidium iodide were obtained from Molecular Probes (Eugene, OR, U.S.A.). The ECL western blotting system was from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, U.S.A.). Caspase-3 assay kit was obtained from Pharmingen–Becton Dickinson Co. (San Diego, CA, U.S.A.). Cell Death Detection ELISA^{PLUS} was obtained from Roche Diagnostics (Indianapolis, IN, U.S.A.). The ERK (PD 98059), p38 (SB 203580), and JNK (SP 600125) inhibitors were obtained from Calbiochem (La Jolla, CA, U.S.A.).

Cell culture and incubations

IMR-32 cells were cultured at 37°C in complex medium [55% (vol/vol) Dulbecco's modified Eagle medium (DMEM) high glucose, 30% (vol/vol) Ham F-12, 5% (vol/vol) α-MEM] supplemented with 10 % (vol/vol) fetal bovine serum (FBS) and antibiotics–antimicotic (50 U/ml penicillin, 50 μg/ml streptomycin, and 0.125 μg/ml amphotericin B) (control medium). Zinc-deficient FBS was prepared by chelation with diethylenetriamine pentaacetic acid as previously described (19, 22).

Cells were grown in control medium until 90% confluence, after which the medium was removed and replaced with control (nonchelated) medium or chelated medium containing 1.5, 5, or 15 μ*M* zinc with or without added catalase (8,000 U/ml). Cells were harvested at different time points, depending on each experiment.

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocols. Total cellular zinc levels were determined by inductively coupled plasma atomic emission spectrometry (Trace Scan; Thermo Elemental, Franklin, MA, U.S.A.) as described (18).

Evaluation of the global concentration of intracellular oxidants and H_2O_2 release

The global concentration of intracellular oxidants was estimated as previously described using DCDCDHF, a nonfluorescent probe, which is oxidized to a fluorescent derivative by endogenous oxidants (22). H_2O_2 release was measured following the oxidation of scopoletin in a reaction catalyzed by 1 U/ml horseradish peroxidase (28). Cells were cultured for 6, 12, or 24 h in the different media. Subsequently, the medium was removed and cells incubated in phenol red-free DMEM for a further 3 h. Fluorescence (365 nm excitation wavelength, 460 nm emission wavelength) was measured using a Perkin–Elmer LS50B luminescence spectrometer (Perkin–Elmer Corp., Norwalk, CT, U.S.A.).

EMSA

Nuclear fractions were isolated basically as previously described (6, 20) with minor modifications (19). For the EMSA, the oligonucleotide containing the consensus sequence for AP-1 was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 50 m*M* Tris-HCl buffer, pH 7.5, containing 4% (vol/vol) glycerol, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 50 m*M* NaCl, and 0.05 mg/ml poly (dI-dC). For the supershift assays, prior to the addition of the labeled nucleotide, samples were incubated in the presence of the corresponding antibodies. The products were separated by electrophoresis in a 4% (wt/vol) nondenaturing polyacrylamide gel using 0.5× TBE (45 m*M* Tris/borate, 1 m*M* EDTA) as the running buffer. The gels were dried and the radioactivity quantitated in a Phosphoimager 640 (Amersham Pharmacia Biotechnology Inc., Piscataway, NJ, U.S.A.).

Northern blot

Total RNA was isolated (5) and electrophoresed on 1% (wt/vol) agarose-formaldehyde gels. After the RNA was transferred to nylon membranes, it was hybridized in 6× SSC (1X: 150 m*M* NaCl and 15 m*M* sodium citrate), 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 5× Denhardt solution, 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, and 20 mg/ml sheared denatured salmon sperm DNA for 18 h at 42°C with *c-jun* cDNA

randomly labeled with [α^{-32}P]dCTP (10^6 cpm/ml of hybridizing solution). Membranes were washed three times with a final stringency of 2× SSC and 0.1% (wt/vol) SDS. Bands were visualized and quantitated using the Phosphoimager 640, and values were normalized to the signal obtained for 28S rRNA.

Transfections

Cells (2.5×10^6 cells) were transfected with LipofectAMINE 2000. As an internal control for transfection efficiency, a vector expressing β-galactosidase (2 μg of of DNA) was cotransfected with the pAP-1-Luc plasmid (1 μg DNA). Twenty-four hours after the transfection was initiated, cells were treated with media containing varying concentrations of zinc (1.5–15 μ*M*). Cells were harvested 12 h later and, after lysis, β-galactosidase and luciferase activities were determined following the manufacturer's protocols.

Western blot

Whole-cell extracts were prepared by lysis in 50 m*M* HEPES, pH 7.4, containing 150 m*M* NaCl, 0.5 m*M* EDTA, 2% (vol/vol) Igepal, phosphatase inhibitors (1 m*M* sodium pervanadate, 100 μ*M* NaF), and protease inhibitors (1 m*M* phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 mg/L pepstatin, and 10 μg/ml aprotinin). Lysates were exposed to one cycle of freezing and thawing, incubated for 30 min on ice, and centrifuged at 15,000 *g* for 30 min. Proteins (25–50 μg/lane) were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. After immunoblotting, the conjugates were detected by enhanced chemiluminescence in a Phosphoimager 640.

To evaluate the phosphorylation of SEK1, JNK, p38, and ERK1/2, antibodies recognizing the phosphorylated/acivated forms of SEK1/MAPK kinase-4 (MKK4 (Thr261), JNK1/2 (Thr183/Tyr185), p38 (Thr180/Tyr182), and ERK1/2 (Tyr204) were used. For these experiments, cell lysates were prepared in SDS sample buffer [62.5 m*M* Tris-HCl buffer, pH 6.8, containing 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 50 m*M* DTT, and 0.01% (wt/vol) bromophenol blue], sonicated for 15 s, and then heated at 95°C for 5 min for further SDS-PAGE.

SAPK/JNK and p38 MAPK assay

After the selective immunoprecipitation of the active kinases, the phosphorylation of c-Jun and ATF-2, the substrates for JNK and p38-catalyzed phosphorylation, respectively, was measured according to the manufacturer's protocols.

Evaluation of cell death by apoptosis

Caspase-3 activity was determined using a commercial assay based on the cleavage of a fluorogenic synthetic tetrapeptide, *N*-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin), specific for caspase-3. The relative fluorescence of the released product was measured (380 nm excitation wavelength, 430 nm emission wavelength). Results were normalized by the protein concentration in the cell lysates, and are expressed as a percentage of the values obtained for the cells incubated in control medium (100%).

The cytoplasmic mono- and oligonucleosomes were evaluated using the Cell Death Detection ELISA^{PLUS}, which quantitates cytoplasmic-histone DNA fragments (mono- and oligonucleosomes), following the manufacturer's protocols. Values (absorbance at 405 nm − absorbance at 490 nm) were referred to the average cell viability values measured with the MTT assay for each group.

Statistical analysis

One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffé, were performed using Statview 5.0 (SAS Institute, Cary, NC, U.S.A.). A p value of <0.05 was considered statistically significant. Values are given as means ± SEM.

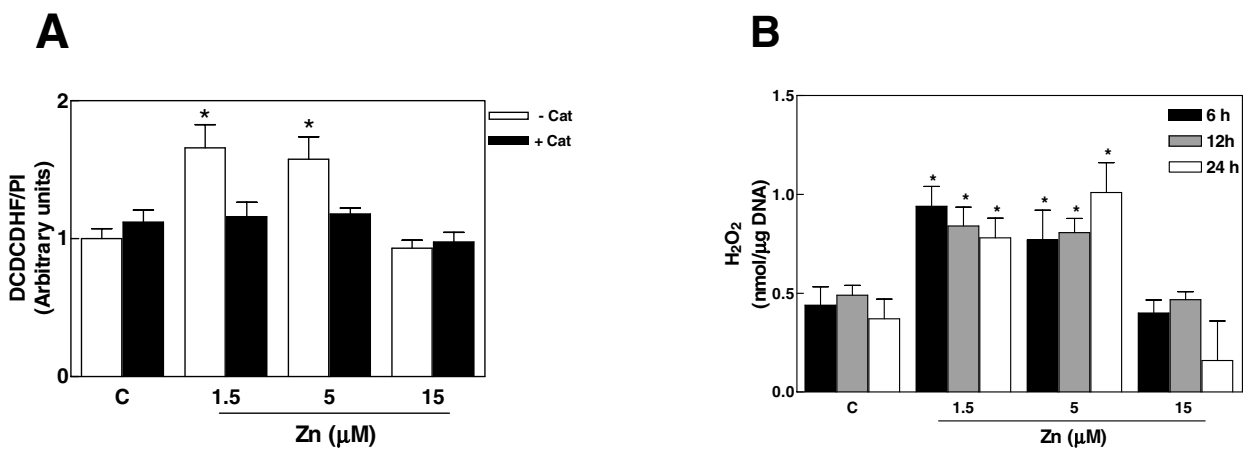

FIG. 1. Effect of catalase on zinc deficiency-induced increases in intracellular oxidant levels and H_2O_2 release. (**A**) Global oxidant levels were evaluated with the probe DCDCDHF in IMR-32 cells after 24 h of incubation in control nonchelated medium (C) or in chelated medium containing 1.5, 5, or 15 μ*M* zinc in the absence (white bars) or presence (black bars) of catalase (Cat). DCDCDHF fluorescence was normalized to the DNA content [propidium iodide (PI) fluorescence]. (**B**) The release of H_2O_2 was measured (for 3 h) after incubating cells for 6, 12, or 24 h in the media with variable concentrations of zinc. H_2O_2 content in the media was normalized by the DNA content in the samples. Results are shown as means ± SEM of five independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.02$, one-way ANOVA test).

RESULTS

Low extracellular zinc concentrations decrease cell zinc concentrations and increase intracellular oxidant levels and H_2O_2 release

IMR-32 cells incubated for 24 h in zinc-deficient media (1.5 and 5 μ*M* zinc) had low ($p < 0.05$, one-way ANOVA) total zinc concentrations (μmol of zinc/mg of protein) (4.14 ± 0.30 and 4.69 ± 0.08 for 1.5 and 5 μ*M* zinc cells, respectively) compared with cells incubated in control or 15 μ*M* zinc media (7.49 ± 0.33 and 6.11 ± 0.87, respectively). After 24 h in culture, cells incubated in zinc-deficient media showed a high DCDCDHF relative fluorescence, compared with cells cultured in control or 15 μ*M* zinc media ($p < 0.02$) (Fig. 1A). The addition of catalase to the media prevented the increase in DCDCDHF fluorescence levels in the 1.5 and 5 μ*M* Zn groups (Fig. 1A). Accordingly, a high release of H_2O_2 to the media was detected at all time points in the zinc-deficient compared with the control and 15 μ*M* Zn groups (Fig. 1B).

Zinc deficiency activates AP-1

To evaluate the effects of zinc deficiency on AP-1-driven transactivation, the expression of endogenous genes (measuring c-Jun mRNA and p53 protein content) and a reporter (luciferase) gene was assessed. After 12 h of incubation in the

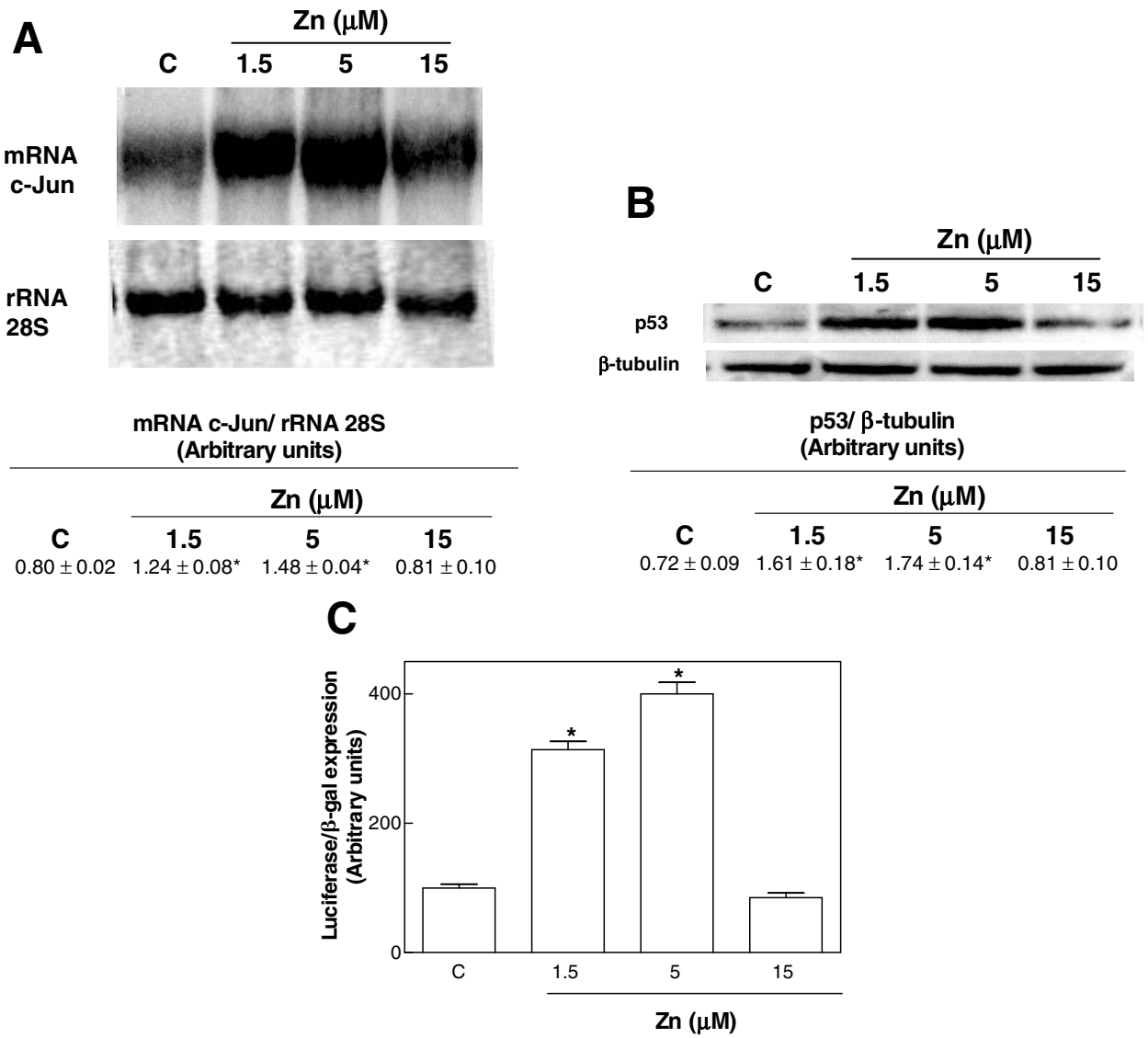

FIG. 2. Influence of zinc on the activation of AP-1: transactivation of AP-1-driven genes. (**A**) c-Jun mRNA levels were measured by northern blot. IMR-32 cells were incubated for 12 h in control nonchelated medium (C) or in chelated medium containing 1.5, 5 μm,or 15 μ*M* zinc. After quantitation, results were expressed as the ratio mRNA c-Jun/rRNA 28S. (**B**) Western blot for p53 after 48 h of incubation. The figure shows a representative image from three independent experiments. After quantitation, results were expressed as the ratio p53/β-tubulin. (**C**) Transactivation of pAP-1-Luc plasmid. Luciferase and β-galactosidase activities were evaluated after 12 h of incubation in the corresponding media. Data are expressed as the ratio luciferase/β-galactosidase activity. Results are shown as means ± SEM of seven independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.001$, one-way ANOVA test).

corresponding media, the mRNA levels of c-Jun measured by Northern blot and corrected for 28S rRNA content were 53–85% higher ($p < 0.01$) in the 1.5 and 5 μ*M* Zn cells than in control and 15 μ*M* Zn cells (Fig. 2A). After 48 h of incubation in the different media, the p53 protein content was 98–145% higher ($p < 0.01$) in the zinc-deficient cells compared with control and 15 μ*M* Zn cells (Fig. 2B).

Transfection assays showed that after 12 h of incubation, luciferase activity was approximately threefold higher in the zinc-deficient cells than in control and 15 μ*M* Zn cells (Fig. 2C).

For the EMSA assays, the specificity of the AP-1–DNA complex was assessed by competition with a 100-fold molar excess of unlabeled oligonucleotides containing the consensus sequence for either AP-1 or OCT-1 (Fig. 3A). At 12 and 24 h, the DNA binding activity of AP-1 in nuclear cell fractions was higher in the zinc-deficient cells than in the control and 15 μ*M* Zn cells (Fig. 3B). There was no increase in AP-1–DNA binding activity in the 1.5 and 5 μ*M* zinc cells when the media contained catalase (Fig. 3C). Consistent with this finding, the p53 content in the zinc-deficient cells treated with catalase was similar to that of controls (data not shown).

The major components of AP-1 were determined by supershift assays with specific antibodies against the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families of proteins. The main AP-1 protein found in IMR-32 cells was c-Jun, with a minor contribution of JunB (data not shown). Consistent with the results obtained by EMSA, high concentrations ($p < 0.05$) of c-Jun were detected by western blot in nuclear fractions prepared from the 1.5 and 5 μ*M* Zn cells (Fig. 3B). The nuclear concentration of c-Jun (arbitrary units) was 0.52 ± 0.09, 1.21 ± 0.12, 1.74 ± 0.11, and 0.69 ± 0.08 for the control, 1.5, 5 and 15 μ*M* zinc cells, respectively.

Zinc deficiency inhibits ERK1/2 MAPK phosphorylation

When cells were incubated in the respective media for 12 h, a lower (by ~44%, $p < 0.05$) ERK1/2 phosphorylation was observed in the zinc-deficient cells compared with the con-

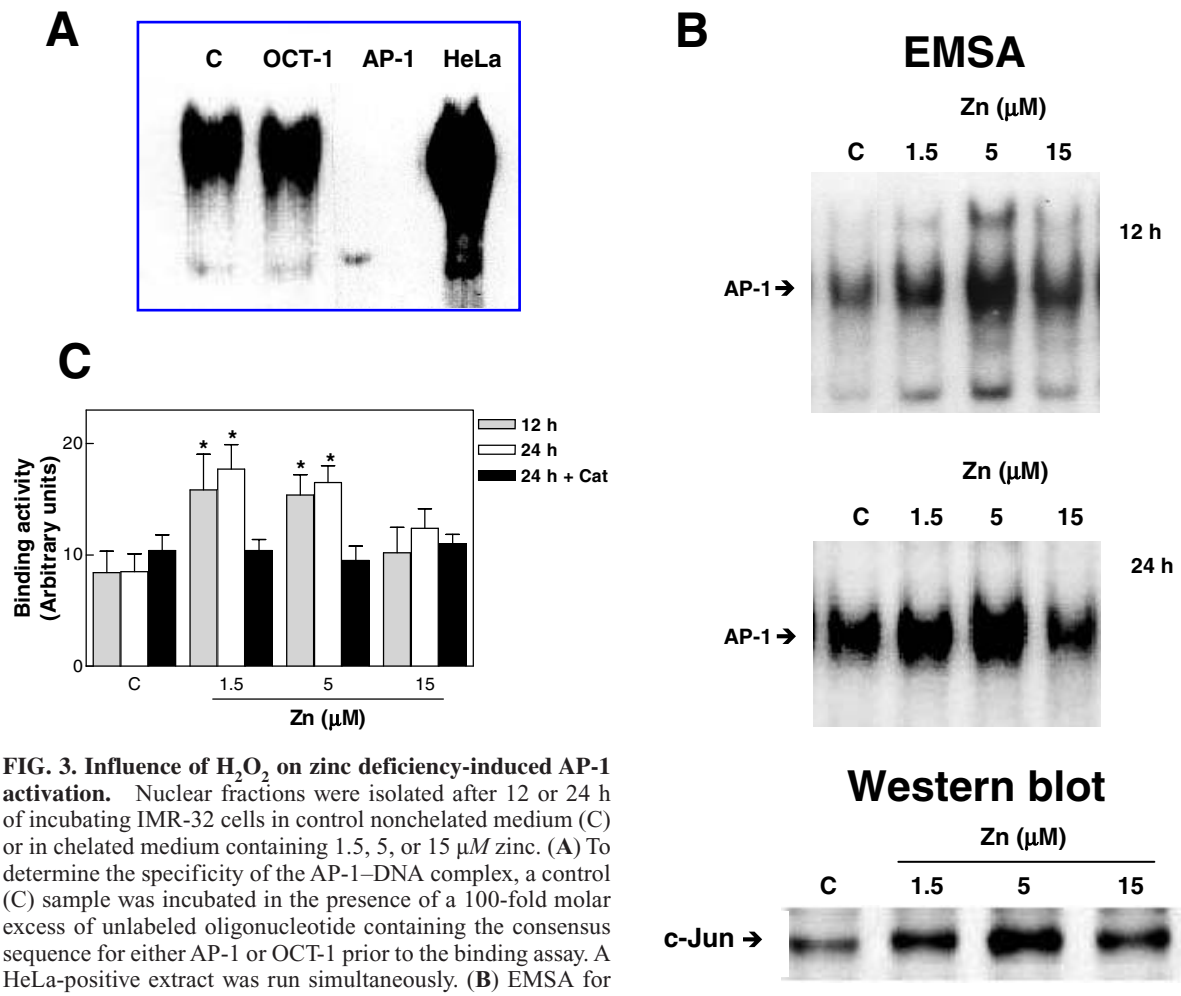

FIG. 3. Influence of H_2O_2 on zinc deficiency-induced AP-1 activation. Nuclear fractions were isolated after 12 or 24 h of incubating IMR-32 cells in control nonchelated medium (C) or in chelated medium containing 1.5, 5, or 15 μ*M* zinc. (**A**) To determine the specificity of the AP-1–DNA complex, a control (C) sample was incubated in the presence of a 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either AP-1 or OCT-1 prior to the binding assay. A HeLa-positive extract was run simultaneously. (**B**) EMSA for AP-1 in nuclear fractions isolated from cells incubated for 12 or 24 h in the corresponding media and western blot for c-Jun. (**C**) After the EMSA assays, bands corresponding to nuclear extracts isolated from cells incubated for 12 h (gray bars) or 24 h in the corresponding media in the absence (white bars) or the presence (black bars) of added catalase (Cat) were quantitated. Results are shown as means ± SEM of seven independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.05$, one-way ANOVA test).

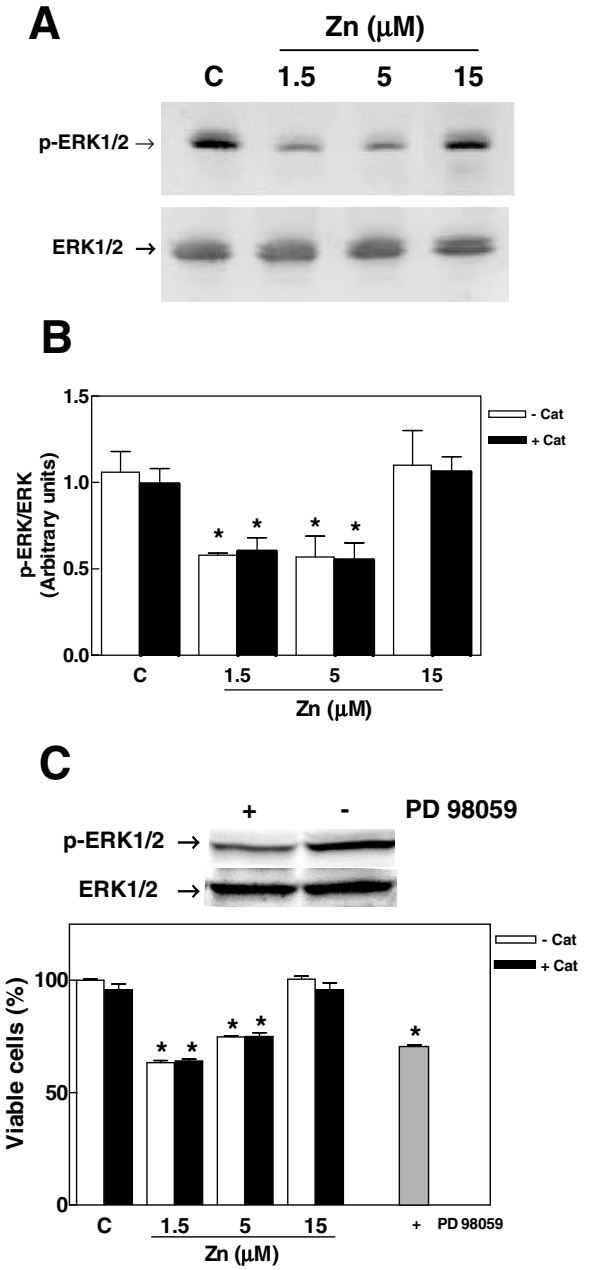

FIG. 4. Influence of H_2O_2 on zinc deficiency-induced changes in the phosphorylation of ERK1/2 and on cell viability. (**A**) Western blots for phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2. Cells were previously synchronized as described under Materials and Methods and further incubated in the media containing variable concentrations of zinc during 12 h for the determination of p-ERK1/2 and ERK1/2. (**B**) After quantitation, the ratio p-ERK/ERK for cells incubated in the media containing variable concentrations of zinc in the absence (white bars) or the presence (black bars) of catalase (Cat) was calculated. Results are shown as means ± SEM of five independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.02$, one-way ANOVA test). (**C**) Cell viability after 24 h of incubation in media containing variable concentrations of zinc in the absence (white bars) or presence (black bars) of catalase. Control cells were also incubated for 24 h with 10 μ*M* PD 98059 (gray bar). (**Upper panel**) Western blot showing p-ERK1/2 inhibition by PD 98059.

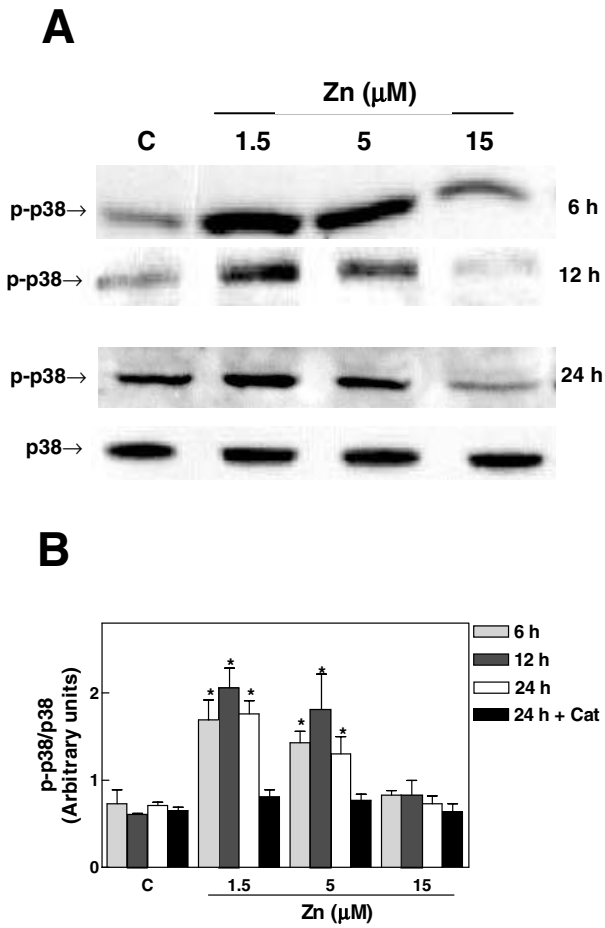

FIG. 5. Influence of H_2O_2 on zinc deficiency-induced changes in the phosphorylation of p38. Total cell fractions were isolated after 6, 12, or 24 h of incubation in control nonchelated medium (C) or chelated medium containing 1.5, 5, or 15 μ*M* zinc. (**A**) Western blots for phosphorylated p38 (p-p38) and p38 for cells incubated in the corresponding media. (**B**) Quantitation of the ratio p-p38/p38 for cells incubated for 6 h (light gray bars), 12 h (dark gray bars) in the media containing variable concentrations of zinc, and for 24 h in the absence (white bars) or the presence (black bars) of added catalase (Cat). Results are shown as means ± SEM of five independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.03$, one-way ANOVA test).

trol and 15 μ*M* zinc groups (Fig. 4A). Reduced ERK1/2 phosphorylation in the 1.5 and 5 μ*M* zinc groups was evident regardless of catalase treatment (Fig. 4B).

After 24 h of incubation, the number of viable cells in the 1.5 and 5 μ*M* Zn groups was lower than that observed in control cells and was not affected by catalase treatment (Fig. 4C). As after 24 h no evidence of apoptotic cell death was observed, the lower number of viable cells can be extrapolated to decreased rates of cell proliferation. The incubation of control cells with an ERK inhibitor (PD 98059) for 24 h caused a 30% decrease in cell viability (Fig. 4C), suggesting the involvement of ERK in IMR-32 cell proliferation.

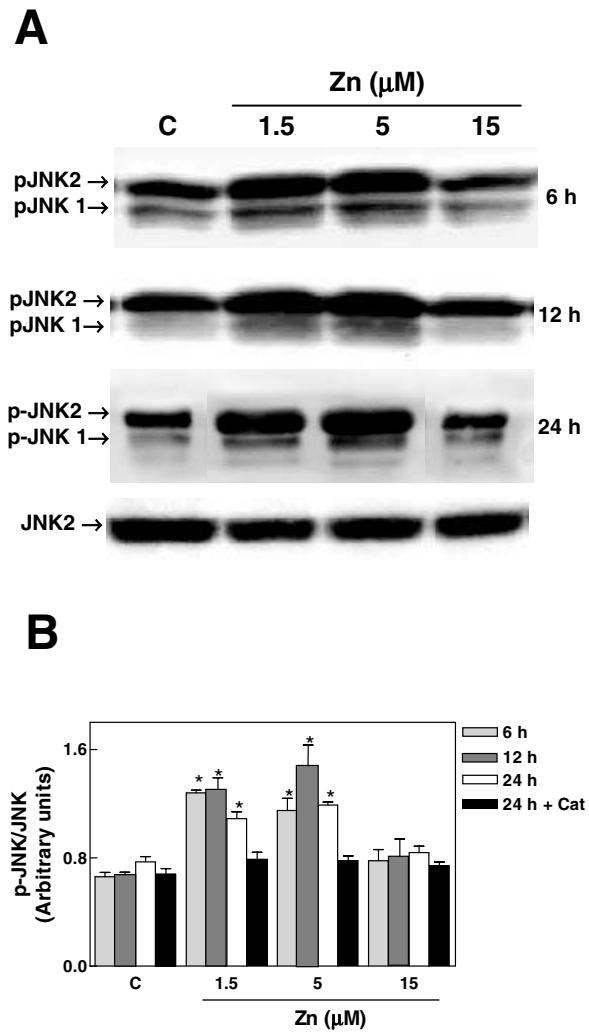

FIG. 6. Influence of H_2O_2 on zinc deficiency-induced changes in the phosphorylation of JNK. Total cell fractions were isolated after 6, 12, or 24 h of incubation in control nonchelated medium (C) or in chelated medium containing 1.5, 5, or 15 μ*M* zinc. (**A**) Western blots for phosphorylated JNK1 (p-JNK1), phosphorylated JNK2 (p-JNK2), and JNK2 for cells incubated in the corresponding media. (**B**) Quantitation of the ratio p-JNK/JNK for cells incubated for 6 h (light gray bars) and 12 h (dark gray bars) in the media containing variable concentrations of zinc, and for 24 h in the absence (white bars) or the presence (black bars) of added catalase (Cat). Results are shown as means ± SEM of five independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.05$, one-way ANOVA test).

Zinc deficiency activates p38 and JNK MAPKs

The kinetics of p38 phosphorylation was followed in IMR-32 cells after 6, 12, and 24 h of incubation in the different media (Fig. 5A). Cells incubated in the zinc-deficient media had a high ($p < 0.01$) level of p-p38 after 6 h of incubation relative to that observed in the control and 15 μ*M* cells (Fig. 5A). This difference persisted after 12 and 24 h of incubation. Zinc deficiency was also associated with an increased phosphorylation of JNK1 (p-JNK1) and JNK2 (p-JNK2) after

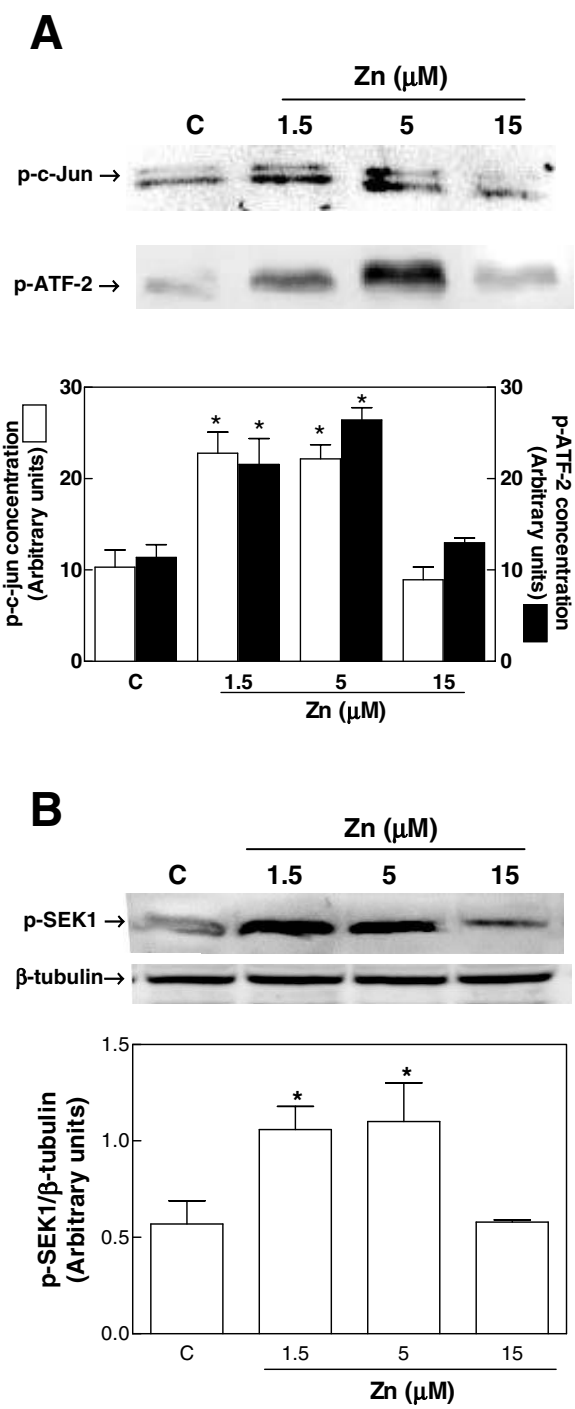

FIG. 7. p38 and JNK activities and the phosphorylation of SEK1/MKK4 are increased with cellular zinc deficiency. (**A**) Kinase activity assays for JNK (p-c-Jun) and p38 (p-ATF-2) were done as described under Materials and Methods, after cells were incubated for 12 h in control nonchelated medium (C) or in chelated medium containing 1.5, 5, or 15 μ*M* zinc. After quantitation, kinase activities for JNK (white bars) and p38 (black bars) are shown as means ± SEM of three independent experiments. (**B**) Western blot for phosphorylated SEK1 (p-SEK1) after 9 h of incubation. After quantitation, the ratio p-SEK1/β-tubulin levels are shown as means ± SEM of three independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.05$, one-way ANOVA test).

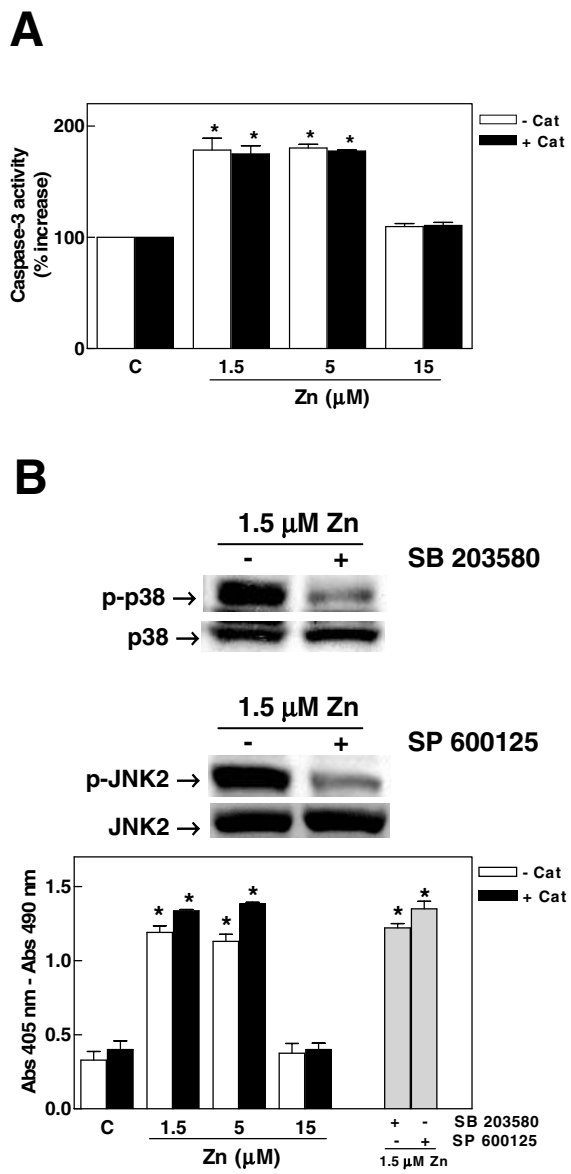

FIG. 8. Effects of catalase on zinc deficiency-induced apoptotic cell death. Cells were incubated in control nonchelated medium (C) or chelated medium containing 1.5, 5, or 15 μ*M* zinc in the absence (white bars) or the presence (black bars) of catalase (Cat). Caspase-3 activity (**A**) and mono- and oligonucleosomes (**B**), after 24 and 48 h of incubation, respectively, were done as described under Materials and Methods. A subset of cells incubated in zinc-deficient medium (1.5 μ*M*) was simultaneously incubated with 10 μ*M* SB 203580 or SP 600125 (gray bars). (**Upper panel**) Western blots showing the inhibition of p38 and JNK phosphorylation in the 1.5 μ*M* Zn cells by SB 203580 and SP 600125, respectively. Results are shown as means ± SEM of four independent experiments. *Significantly different compared with C and 15 μ*M* Zn groups ($p < 0.01$, one-way ANOVA test).

6 h of incubation in the corresponding media (Fig. 6A). Elevated phosphorylation was also evident at the 12- and 24-h time points.

The activity of p38 and JNK was measured after 12 h of incubation (Fig. 7A). Consistent with the observed increase in p38 and JNK phosphorylation, activities of both kinases were higher ($p < 0.05$) in the cells incubated in the zinc-deficient media than in the control and 15 μ*M* Zn cells (Fig. 7A).

An upstream event in the JNK and p38 cascades was next evaluated. The phosphorylation of the MAPK kinase (SEK1/MKK4) was measured after 9 h of incubation in the different media (Fig. 7B). SEK1/MKK4 phosphorylation, was significantly higher (90–100%, $p < 0.01$) in the zinc-deficient cells (1.5 and 5 μ*M* Zn) than in the control and 15 μ*M* Zn cells.

To investigate the involvement of H_2O_2 on p38 and JNK activation in the zinc-deficient cells, the effect of catalase treatment on p38 and JNK phosphorylation was studied. Increased levels of phosphorylation of these kinases were not observed in the 1.5 and 5 μ*M* Zn cells when catalase was added to the incubation media (Figs. 5B and 6B).

Increased production of H_2O_2 is not essential for zinc deficiency-induced cell apoptosis

After 24 h of incubation in the different media, a higher activity of caspase-3 was measured in the zinc-deficient cells than in the control and 15 μ*M* Zn cells; the presence of catalase did not prevent this increase (Fig. 8A). Consistently, after 48 h of incubation, high concentrations of mono- and oligonucleosomes were found in the zinc-deficient cells, regardless of the absence or presence of catalase (Fig. 8B). These results suggest that an oxidant-mediated activation of JNK, p38, and AP-1 is not essential for the triggering of apoptosis secondary to low cellular zinc levels. In support of this, the inhibition of p38 and JNK by SB 203580 and SP 600125, respectively, did not prevent DNA fragmentation in the zinc-deficient cells.

DISCUSSION

This study showed that a decrease in cellular zinc leads to a differential modulation of MAPKs. Zinc deficiency was found to be associated with a low activity of ERK1/2 and high activities of MAPKs p38 and JNK1/2. An increase in cellular H_2O_2 triggered the activation of JNK and p38 and subsequently of AP-1 in the zinc-deficient cells. Although AP-1 can be a proapoptotic signal, zinc deficiency-induced apoptosis in IMR-32 cells was not mediated by the JNK/p38/AP-1 cascade.

The increase in cell oxidants observed in the zinc-deficient cells was prevented by catalase, indicating that H_2O_2 is the major oxidant species produced secondary to zinc deficiency. Exogenous catalase has been previously shown to reduce cellular H_2O_2 concentrations (7, 24) and to inhibit both endogenous H_2O_2-mediated MAPK activation (3) and apoptotic cell death (4). The high AP-1-DNA binding activity found in the zinc-deficient IMR-32 cells was triggered by H_2O_2 and led to a subsequent increase in the transactivation of AP-1-dependent genes. Although other oxidants, such as reactive nitrogen species (13), could be generated as a consequence of de-

creased cellular zinc, the inhibitory action of catalase on AP-1 activation indicates that H_2O_2 is the signal triggering AP-1.

The activation of AP-1 by H_2O_2 is thought to occur primarily through the activation of the stress-responsive MAPKs p38 and JNK. Different mechanisms have been proposed to explain this oxidant-mediated activation; such as the inhibition of MAPK phosphatases (12) and the activation of apoptosis signal-regulating kinase-1 (ASK1), potentially through the regulation by oxidants of the interaction between thioredoxin and ASK1 (25). In the zinc-deficient cells, the activation of AP-1 was mainly driven by the H_2O_2-mediated activation of p38 and JNK, because ERK1/2 phosphorylation was low.

It has been proposed that H_2O_2 can, as a second messenger, increase the activity of tyrosine kinase receptors leading to the activation of the Ras-ERK1/2 cascade (9). In vascular smooth muscle cells, H_2O_2 activates ERK1/2 through Ca^{2+}/calmodulin-dependent pathways (2). However, a reduction in the levels of ERK1/2 phosphorylation in the zinc-deficient cells was observed that was independent of H_2O_2 and that could be related to the requirement of zinc for cell growth (1). Furthermore, the activation of the MAPK pathway Raf/MEK/ERK1/2 by mitogenic signals and growth factors (27) and the present finding that ERK1/2 inhibition by PD 98059 decreases the number of viable IMR-32 cells support the hypothesis that ERK1/2 inhibition could constitute a factor in the decreased cell proliferation associated with zinc deficiency.

Through the activation of AP-1, H_2O_2 could be involved in processes of proliferation or apoptotic cell death depending, among other factors, on the cell type and the intensity and duration of the stimuli (9, 11, 15). Zinc deficiency induces apoptosis in both cell cultures and whole animal models, but the exact mechanisms are still unknown (8, 10, 14, 30, 31). Given the evidence that oxidants and antioxidants can modulate apoptosis, combined with information on the role of AP-1 in apoptotic cell death (26), we investigated if H_2O_2-driven AP-1 activation could be one signal involved in the triggering of apoptosis in zinc deficiency. Caspase-3 activity, considered an irreversible step in the apoptotic cascade, and the levels of cytosolic mono- and oligonucleosomes, formed secondarily to DNA fragmentation, were elevated in the zinc-deficient cells regardless of the absence or presence of catalase. Thus, an increase in apoptosis is observed in the zinc-deficient cells, which is independent of AP-1 activation. Zinc deficiency-induced ERK1/2 inhibition and associated decrease in cell proliferation could contribute to cell apoptosis because ERK1/2 inhibition by PD 98059 in control IMR-32 cells induced a significant increase in apoptosis after 48 h (data not shown).

In summary, the obtained results contribute to elucidate the upstream mechanisms involved in the activation of AP-1 associated with zinc deficiency. IMR-32 cells cultured in zinc-deficient media are characterized by a rapid increase in cellular oxidative stress and elevated levels of H_2O_2. A differential modulation of MAPKs was observed in association with zinc deficiency. Through H_2O_2-independent pathways, a decrease in cellular zinc causes a reduction in ERK1/2 MAPK activation. The increase in intracellular H_2O_2 is the signal leading to the activation of p38 and JNK MAPKs, with the subsequent triggering of the activation of transcription factor AP-1. Furthermore, although AP-1 activation can lead to cell apoptosis in several physiological and pathological conditions, we present evidence that H_2O_2-induced p38/JNK/AP-1 activation is not a pathway involved in the IMR-32 cell apoptosis induced by zinc deficiency.

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ABBREVIATIONS

ANOVA, analysis of variance; AP-1, activator protein-1; ASK1, apoptosis signal-regulating kinase-1; ATF-2, activating transcription factor-2; DCDCDHF, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCT-1, octamer binding transcription factor-1; PAGE, polyacrylamide gel electrophoresis; p-ERK, phosphorylated ERK; p-JNK, phosphorylated JNK; p-p38, phosphorylated p38; SAPK, stress-activated protein kinase; SDS, sodium dodecyl sulfate; SEK1, SAPK kinase-1.

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