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Low Intracellular Zinc Impairs the Translocation of Activated NF-κB to the Nuclei in Human Neuroblastoma IMR-32 Cells*

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In the current work, we studied how variations in extracellular zinc concentrations modulate different steps involved in nuclear factor κB (NF-κB) activation in human neuroblastoma IMR-32 cells. Cells were incubated in media containing varying concentrations of zinc (1.5, 5, 15, and 50 μM). Within 3 h, the intracellular zinc content was lower in cells exposed to 1.5 and 5 μM, compared with the other groups. Low intracellular zinc concentrations were associated with the activation of NF-κB, based on high levels of IκBα phosphorylation, low IκBα concentrations, and high NF-κB binding activity in total cell fractions. However, the active dimer accumulated in the cytosol, as shown by a low ratio of nuclear/cytosolic NF-κB binding activity. This altered nuclear translocation was accompanied by a decreased transactivation of an endogenous NF-κB-driven gene (*ikba*) and of a reporter gene (pNF-κB-luc). In cells with low intracellular zinc concentrations, a low rate of *in vitro* tubulin polymerization was measured compared with the other groups. We conclude that low intracellular zinc concentrations induce tubulin depolymerization, which may be one signal for NF-κB activation. However, NF-κB nuclear translocation is impaired, which inhibits the transactivation of NF-κB-driven genes. This could affect cell survival, and be an important factor in certain zinc deficiency-associated pathologies.

The mature brain is relatively well protected from the deleterious effects of zinc deficiency (1). However, the developing brain can be highly sensitive to a deficit of this nutrient for several reasons, including the need of zinc for appropriate cell differentiation, migration, and growth. Consistent with this, developmental zinc deficiency is characterized by a high frequency of brain and eye malformations, including agenesis and dysmorphogenesis of the brain, spinal cord, eye, and olfactory tract (2, 3). In addition to gross structural malformations, zinc deprivation during critical developmental periods can result in altered emotionality and food motivation early in life (4).

Although numerous structural defects have been reported to occur as a consequence of embryonic and fetal zinc deficiency (5), the mechanisms underlying these defects are poorly understood. However, it has been reported that gestational zinc deficiency for a period of time as short as 4 days can result in excessive cell death in the conceptus, particularly in the neural crest cell region (5). Based on histological evidence, the excessive cell death may be through programmed cell death (apoptosis).

Rel/NF-κB[1] transcription factors are activated by multiple signals, and regulate the expression of numerous genes. Work to date suggests that this transcription factor is involved in the regulation of the immune and stress response, in cell cycle progression, in the decision of cells to undergo apoptosis and in the maintenance of cell structure and the nearby environment (6). Known members of Rel/NF-κB family of proteins include c-Rel, RelB, RelA (p65), p50, and p52. The activity of the Rel/NF-κB homo- and heterodimers is regulated by their interaction with inhibitory IκB proteins, which anchor the transcription factor to the cytosol (7). One of the best described interactions is that of IκBα, which prevents the translocation of NF-κB to the nuclei and its binding to DNA. In general, activation is mediated by the phosphorylation of two conserved serines (Ser-32 and Ser-36 in human IκBα) in IκB by specific IκB kinases, which targets IκBα for ubiquitination and degradation by the proteasome (8).

We previously reported that there is a reduction in NF-κB binding activity in nuclear extracts of 3T3 cells after they are incubated in media containing low concentrations of zinc (0.5 and 5 μM) for 24 h (9). Similarly, a low NF-κB binding activity was observed in nuclear extracts obtained from testes of developing male rats fed low zinc diets for 1 week (10). These results suggest that zinc can modulate NF-κB activation; however, the mechanism(s) underlying this effect of a deficit of zinc are unclear.

Alterations in the cytoskeletal network could affect the translocation of transcription factors, such as NF-κB, that reside in the cytosol in an inactive form, which, after activation, require the translocation of the active form to the nuclei (11). Our previous findings of impaired tubulin polymerization in brains obtained from zinc-deficient animals (12–14) led us to postulate that low intracellular zinc could result in a defect in the transportation of NF-κB from the cytosol to the nucleus.

A series of papers have demonstrated, in certain cell types,

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[1] The abbreviations used are: NF-κB, nuclear factor κB; *n* Zn, medium containing *n* μM zinc; ANOVA, analysis of variance; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; TSQ, *N*-6-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

that the activation of NF-κB can protect cells from apoptosis, suggesting a role for NF-κB in cell survival (15–17). Thus, we reasoned that the brain teratogenicity associated with gestational zinc deficiency could be caused, in part, by increased cell death by apoptosis secondary to an impairment in NF-κB activation.

To begin to study the possible mechanisms underlying the modulation of NF-κB by zinc, we exposed human neuroblastoma IMR-32 cells to media containing different concentrations of zinc (1.5–50 μM). In the current work, we characterized the effect of these media on the DNA binding activity of NF-κB in these cells, as well as on several different steps in NF-κB activation, including the transactivating capacity of endogenous NF-κB-driven gene (*ikba*) (18–20) and a reporter gene. Data obtained from this study suggest that low intracellular zinc concentrations impair tubulin assembly, which may be one signal for NF-κB activation. We suggest that zinc tubulin disassembly contributes to the altered NF-κB nuclear translocation and a subsequent inhibition of NF-κB-dependent gene transactivation.

EXPERIMENTAL PROCEDURES

Materials—IMR-32 cells were obtained from the American Type Culture Collection (Rockville, MA). Cell culture media and reagents and LipofectAMINE™ 2000 were obtained from Invitrogen. The oligonucleotides containing the consensus sequences for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and OCT-1, the reagents for the EMSA 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). The PathDetect NF-κB cis reporting system was obtained from Stratagene (La Jolla, CA). The cDNA for IκBα was a gift from Dr. E. Arzt (University of Buenos Aires, Buenos Aires, Argentina). Antibodies for RelA, p50, p52, IκBα, and β tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA); antibody for phospho-IκBα was from Cell Signaling Technology (Beverly, MA). Polyvinylidene difluoride and Zeta-Probe membranes were obtained from Bio-Rad (Hercules, CA), and Chroma Spin-10 columns were obtained from CLONTECH (Palo Alto, CA). *N*-6-(6-Methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ) was obtained from Molecular Probes (Eugene, OR). The ECL Western blotting system was from Amersham Biosciences. The APO-BRDU™ kit was from BD PharMingen (San Diego, CA). Lactacystin was obtained from Calbiochem (La Jolla, CA). All other reagents were the highest quality available and were purchased from Sigma.

Cell Culture—IMR-32 cells were cultured at 37 °C in Complex medium (55% DMEM high glucose, 30% Ham's F-12, 5% α-minimal essential medium) supplemented with 10% fetal bovine serum (FBS), and antibiotic-antimycotic (50 units/ml penicillin, 50 μg/ml streptomycin, and 0.125 μg/ml amphotericin B).

Zinc-deficient FBS was prepared by chelation with diethylenetriamine pentaacetic acid as previously described (9). The chelated FBS was subsequently diluted with complex medium to a final concentration of 3 mg/ml protein to match the protein concentration of the control nondialyzed media (10% FBS). The zinc concentration of the zinc-deficient medium was 1.5 μM, and portions of this medium were supplemented with $ZnCl_2$ to reach concentrations of 5, 15, and 50 μM.

Cells were grown in control nondialyzed medium (complex medium containing 10% nonchelated FBS) until 90% confluence, after which the medium was removed and replaced with control medium or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn), 15 (15 Zn), or 50 (50 Zn) μM zinc. Cells were harvested at 3, 6, 12, 24, or 48 h in culture.

Determination of Intracellular Zinc Levels—Cells (1.2×10^6) were incubated in the corresponding media for 3–48 h. At the corresponding time points, the medium was decanted and cells were rinsed with warm DMEM and added with 1 ml of DMEM containing 25 μM TSQ. Cells were dispersed and incubated at 37 °C in the dark for 15 min. Cells were transferred to 1.5-ml conical tubes and centrifuged at $800 \times g$ for 10 min. The cell pellet was rinsed twice with PBS and finally resuspended in 0.2 ml of PBS containing 0.1% Igepal. After a brief sonication, the fluorescence at 480 nm ($\lambda_{exc} = 365$) was measured. To evaluate the DNA content, samples were incubated with 50 μM propidium iodide. After incubating for 20 min at room temperature, the fluorescence ($\lambda_{exc} = 538$, $\lambda_{em} = 590$) was measured. Results are expressed as the ratio TSQ fluorescence/propidium iodide fluorescence.

Determination of Total Zinc, Copper, and Iron—After 24 h in the respective media, the medium was discarded and cells (15×10^6) were scrapped and rinsed three times with warm PBS. After centrifuging at $800 \times g$ for 10 min at room temperature, the pellet was frozen at −80 °C and, after thawing, it was resuspended to a final volume of 0.4 ml. After a brief sonication, an aliquot was taken for the determination of protein concentration and the rest of the sample was wet ashed with 16 M nitric acid (Baker's Instra-analyzed: J.T. Baker, Philipsburg, NJ). Concentrations of zinc, copper, and iron were determined by ICP-AES (Trace Scan; Thermo Elemental, Franklin, MA). Certified reference solutions (QC 21, Spec CentriPrep, Metuchen, NJ) were used to generate standard curves for each element. A sample of a National Bureau of Standards Bovine Liver (SRM 1577; United States Department of Commerce, National Bureau of Standards, Washington, DC) was included with the samples to ensure accuracy and reproducibility.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear and cytosolic fractions were isolated as previously described (21, 22). At the corresponding time points, the medium was discarded and cells were rinsed with PBS and scraped. After centrifugation at $800 \times g$ for 10 min, the pellet (20×10^6 cells) was resuspended in 200 μl of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% Igepal), incubated for 10 min at 4 °C, and centrifuged for 1 min at $12,000 \times g$. The supernatant fraction was removed, and the nuclear pellets were resuspended in 60 μl of buffer B (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF). Samples were incubated for 20 min at 4 °C and centrifuged at $10,000 \times g$ for 15 min at 4 °C. The supernatant was transferred to a new tube and diluted in 45 μl of buffer C (20 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF). Protein concentration was determined by the method of Bradford (23), and samples were stored at −80 °C.

For the EMSA, the oligonucleotide containing the consensus sequence and NF-κB was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 0.25 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 (RelA, p50, or p52). The products were separated by electrophoresis in a 4% nondenaturing polyacrylamide gel using 0.5× TBE (45 mM Tris borate, 1 mM EDTA) as the running buffer. The gels were dried and the radioactivity quantitated in a PhosphorImager 640 (Amersham Biosciences).

Western Blot Analysis—For the preparation of total cell extracts, cells (20×10^6 cells) were rinsed with PBS, scraped, and centrifuged. The pellet was rinsed with PBS and resuspended in 200 μl of 50 mmol/liter HEPES, pH 7.4, 125 mM KCl, which contained protease inhibitors and 2% Igepal. The final concentration of the inhibitors was 0.5 mmol/liter PMSF, 1 mg/liter leupeptin, 1 mg/liter pepstatin, 1.5 mg/liter aprotinin, 2 mg/liter bestatin, and 0.4 mM sodium pervanadate. Samples were exposed to one cycle of freezing and thawing, incubated at 4 °C for 30 min, and centrifuged at $15,000 \times g$ for 30 min. The supernatant was decanted, and protein concentration was measured (23).

Aliquots of total, nuclear, or cytosolic fractions containing 25–50 μg of protein were separated by reducing 10–12.5% polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride membranes. Molecular weight standards (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were run simultaneously. Membranes were blotted overnight in 5% nonfat milk, incubated in the presence of corresponding antibodies for RelA, p50, phospho-IκBα, or IκBα (1:1000 dilution) for 90 min at 37 °C. After incubation, for 90 min at room temperature, in the presence of the secondary antibody (horseradish peroxidase-conjugated) (1:10,000 dilution), the conjugates were visualized by chemiluminescence detection in a PhosphorImager 640.

Northern Blot—Total RNA was isolated using a solution of acid guanidinium thiocyanate-phenol-chloroform (24) and electrophoresed on 1% agarose-formaldehyde gels. After transferring the RNA to nylon membranes, it was hybridized in 6× SSC (1× SSC: 150 mM NaCl and 15 mM sodium citrate), 0.5% sodium dodecyl sulfate, 5× Denhardt's solution, 50% formamide, 10% dextran sulfate, and 20 mg/ml sheared denatured salmon sperm DNA for 18 h at 42 °C with IκBα cDNA randomly labeled with [γ-^{32}P]dCTP (10^6 cpm/ml hybridizing solution). Membranes were washed three times with a final stringency of 2× SCC (5 min at room temperature, followed by two washes of 15 and 30 min at 65 °C). Bands were visualized and quantitated using the PhosphorImager 640, and values were normalized to the signal obtained for 28 S mRNA.

Transfections—IMR-32 cells (2.5×10^6 cells) were transfected with

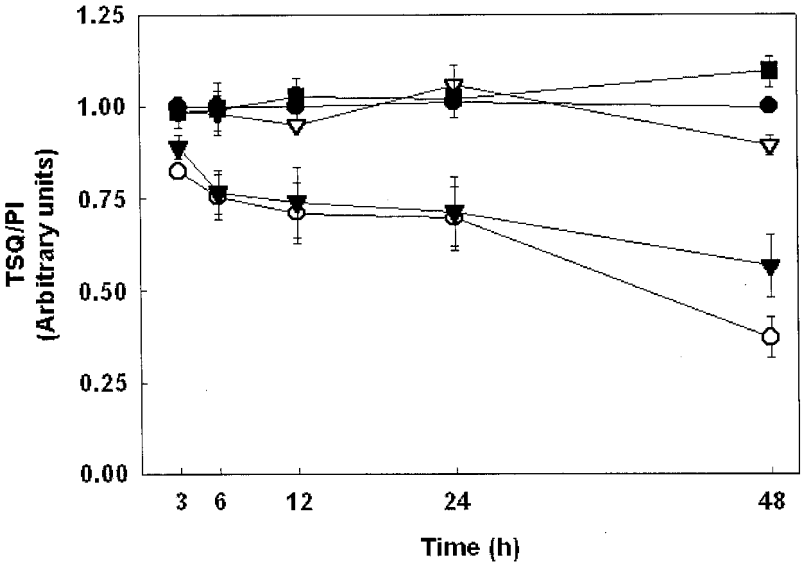

FIG. 1. **Intracellular TSQ-reactive zinc levels vary rapidly with extracellular zinc concentrations.** Undifferentiated IMR-32 neuroblastoma cells were incubated for 3–48 h in control nonchelated media (●) or in chelated media containing 1.5 (○), 5 (▼), 15 (▽), or 50 (■) μM zinc. The intracellular zinc concentration was determined as described under "Experimental Procedures." TSQ fluorescences (*RF*, relative fluorescence) was normalized to the propidium iodide fluorescence in each sample to correct for differences in cell number. Values are shown as the means of at least four independent experiments.

LipofectAMINE™ 2000 according to the protocols of the manufacturer. As an internal control for transfection efficiency, a vector expressing β-galactosidase (2 μg of DNA) was co-transfected with the pNF-κB-Luc plasmid (1 μg of DNA). After 24 h of initiated transfection, cells were treated with the media containing varying concentrations of zinc. Cells were harvested 24 h later, and, after lysis, β-galactosidase and luciferase activities were determined following the protocols of the manufacturer.

In Vitro Microtubule Assembly—Cells (35×10^6) were rinsed three times with 0.1 M Pipes buffer, pH 7.0. Cells were manually homogenized using a glass homogenizer to a final volume of 0.4 ml. The homogenate was incubated for 30 min at 4 °C to allow microtubule depolymerization and then centrifuged at 100,000 × *g* for 30 min at 4 °C. The supernatant was decanted, 200-μl aliquots were placed in a 96-well plate, and tubulin assembly was followed as the increase in absorbance at 340 nm for 90 min. The polymerization was started when the samples were placed at 37 °C in a PerkinElmer HTS 7000 Plus Bio Assay Reader (PerkinElmer Life Sciences) and was followed for 90 min.

Evaluation of Apoptosis—Cells were incubated for 24 h in control media and in the absence or the presence of 10 μM lactacystin. Apoptosis was evaluated by measuring DNA strand breaks using the APO-BRDU™ kit following the protocol of the manufacturer. The assay is based in the labeling of the 3′-hydroxyl termini of DNA strand breaks with bromolated deoxyuridine triphosphates, reaction catalyzed by the terminal deoxynucleotidyltransferase enzyme. These sites were identified by staining with a fluorescein-labeled anti-bromodeoxyuridine antibody, and cells were analyzed on a Becton-Dickinson flow cytometer.

Statistical Analysis—One-way analysis of variance (ANOVA) with subsequent *post hoc* comparisons by Scheffe were performed using Statview 512+ (Brainpower Inc., Calabazas, CA). A p value < 0.05 was considered statistically significant. Values are given as means ± S.E.

RESULTS

Variations in Extracellular Zinc Concentration Affect Intracellular Zinc Levels in Neuroblastoma IMR-32 Cells—To assess whether variations in extracellular zinc concentrations were associated with changes in intracellular zinc pools, we followed changes in intracellular zinc concentrations over a 48-h period. TSQ is a lipid-soluble probe that can cross membranes and react with intracellular zinc. TSQ can bind to membrane zinc, and possibly to loosely bound zinc; both pools of zinc that are thought to be rapidly available for cellular requirements. Within 3 h intracellular zinc dropped in the 1.5 and 5 Zn groups (Fig. 1). After 24 h, a 30–35% decrease in TSQ fluorescence was observed in the 1.5 and 5 Zn groups; by 48 h, values were decreased by 70 and 50%, respectively, compared with controls (Fig. 1). The amount of TSQ fluorescence in the 1.5 and 5 Zn groups was significantly lower ($p < 0.02$, one-way ANOVA test) than in the other three groups at all the time points studied. Intracellular zinc concentrations in cells incubated for 3–48 h in the chelated media containing 15 and 50 μM Zn, and the nonchelated control media, were similar.

After 24 h, total intracellular zinc concentrations (nmol/mg protein) were lower ($p < 0.01$) in the 1.5 Zn (4.1 ± 0.3) and 5 Zn (4.6 ± 0.1) cells compared with the control (7.2 ± 0.3), 15 Zn (6.1 ± 0.9), and 50 Zn (6.4 ± 0.2) groups. Cellular copper and iron concentrations were similar among the groups.

Exposure to the low zinc media did not affect the number of viable cells (determined by trypan blue exclusion) at either the 6- or 12-h time point (data not shown), but by 24 h, there was a decrease in the number of viable cells in the 1.5 and 5 μM Zn groups (35 and 28%, respectively).

Low Intracellular Zinc Levels Activate NF-κB—To investigate the total level of NF-κB activation, we measured the NF-κB-DNA binding activity in total cell fractions by EMSA, the phosphorylation, and the concentration of the inhibitory peptide IκBα by Western blot.

The specificity of the NF-κB-DNA complex was assessed by competition with a 100-fold molar excess of unlabeled oligonucleotides containing the consensus sequence for either NF-κB or OCT-1 (Fig. 2*A*). At 24 h, the DNA binding activity of NF-κB in total cell fractions was higher in the 1.5 and 5 Zn cells than in the control, 15 Zn, and 50 Zn cells (Fig. 2, *B* and *C*). One of the required steps in the activation of NF-κB is, in general, the phosphorylation and degradation of an inhibitory IκB protein that prevents the translocation and binding of the active NF-κB dimer to DNA. IκBα concentration, and the extent of IκBα phosphorylation, were determined by Western blot in total cell fractions (Fig. 3*A*). In agreement with the EMSA observations, the level of IκBα phosphorylation was 52 and 44% higher in the 1.5 and 5 Zn cells, respectively, compared with the control group. At 24 h, the concentration of IκBα measured by Western blot was ~40% lower in the 1.5 and 5 Zn cells than in the control and 15 Zn groups (Fig. 3*B*). At 48 h, a similar pattern was observed, the concentration of IκBα was 30–60% lower in the 1.5 and 5 Zn cells than in the control and 15 Zn cells (data not shown). The content of p- IκBα and IκBα was referred to p50, as its concentration did not vary among groups (Fig. 3*A*). The ratio RelA/p50 was close to 1, and was similar in all the groups.

Low Intracellular Zinc Concentrations Are Associated with an Impaired Translocation of Activated NF-κB to the Nuclei—We previously observed, in testes from zinc-deficient rats (10) and in 3T3 cells exposed to zinc-deficient media (9), that a low zinc status was associated with a low nuclear binding

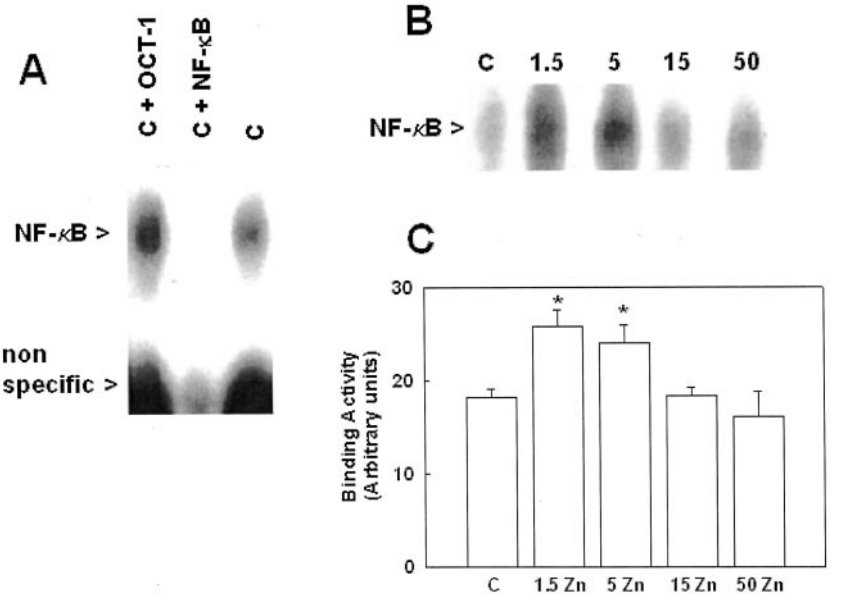

FIG. 2. **NF-κB-DNA binding activity in total fractions from IMR-32 cells.** Fractions were isolated after 24 h of exposure to control nonchelated media (*C*) or to chelated media containing 1.5 (*1.5 Zn*), 5 (*5 Zn*), 15 (*15 Zn*), or 50 (*50 Zn*) μM zinc. *A*, to determine the specificity of the NF-κB-DNA complex, a control (*C*) sample was incubated in the presence of a 100-fold molar excess of unlabeled oligonucleotides containing the consensus sequence for either NF-κB or OCT-1 prior to the binding assay. *B* and *C*, EMSA of the total cell fractions (*B*) and quantitation of the bands (*C*). Results are shown as means ± S.E. of five independent experiments. *, significantly different from the control, 15 Zn, and 50 Zn groups ($p < 0.05$, one-way ANOVA test).

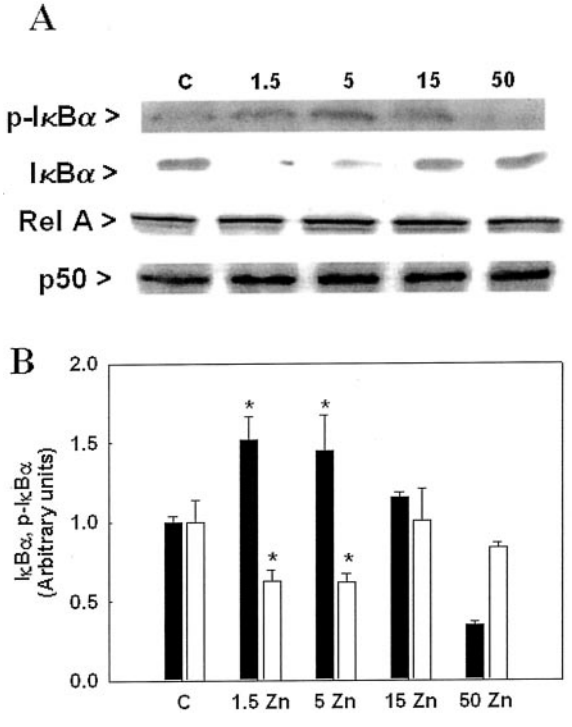

FIG. 3. **Phosphorylated IκBα, IκBα, p50, and RelA levels in total fractions isolated from IMR-32 cells.** Fractions were isolated after 24 of exposure to control nonchelated media (*C*) or to chelated media containing 1.5 (*1.5 Zn*), 5 (*5 Zn*), 15 (*15 Zn*), or 50 (*50 Zn*) μM zinc. *A*, Western blots for phosphorylated IκBα (*p- IκBα*), IκBα, p50, and RelA. *B*, after quantitation, phosphorylated IκBα (*full bars*) and IκBα (*empty bars*) concentrations were referred to the p50 content. Results are shown as means ± S.E. of four independent experiments. *, significantly different compared with control, 15 Zn, and 50 Zn groups ($p < 0.001$, one-way ANOVA test).

activity of NF-κB. In agreement with the above, in IMR-32 cells, after 24 h of incubation in the corresponding media, a reduced ($p < 0.001$) NF-κB nuclear binding activity was observed in the 1.5 and 5 Zn (69 and 57% reduction, respectively) cells, relative to the control group (Fig. 4). Cells in the chelated media supplemented with 15 and 50 μM Zn showed values similar to those for the cells exposed to control nonchelated media. In contrast to the above, NF-κB binding activity in the cytosolic fraction was significantly higher ($p < 0.001$) in the 1.5 and 5 Zn cells than in the other groups (Fig. 4). The ratio of cytosolic/nuclear NF-κB binding activity was ~6 and 4 times higher in the 1.5 and 5 Zn cells, respectively, than in the other three groups.

We investigated whether the low nuclear binding of NF-κB to DNA could be caused by a requirement of zinc for an appropriate NF-κB-DNA interaction. Nuclear fractions from cells incubated in control and chelated media containing 1.5 μM zinc were added during the binding reaction with variable zinc concentrations (0.1–10 μM). The addition of zinc did not modify the nuclear binding of NF-κB to DNA (data not shown).

Alterations in the Translocation of Activated NF-κB to the Nuclei Are Also Observed after Evaluating the Nuclear Concentration of p50 and RelA—To further characterize a possible association between low intracellular zinc concentrations and alterations in the nuclear translocation of the active NF-κB, we evaluated the concentrations of p50 and RelA in the nuclear fractions. The members of the Rel/NF-κB proteins present in the active NF-κB in IMR-32 cells were first characterized by an EMSA supershift assay. A control nuclear fraction was incubated in the presence of antibodies against RelA, p50, or p52, prior to the binding assay. The supershift assay showed that, in IMR-32 cells, the active NF-κB dimer is composed by RelA and p50 proteins (Fig. 5*A*).

The content of RelA and p50 was determined by Western blot in the nuclear (Fig. 5*B*) and cytosolic fractions. A low ratio nuclear/cytosolic content for RelA and p50 was observed in the 1.5 and 5 Zn cells compared with that observed in the control, 15 Zn, and 50 Zn groups (Fig. 5*C*).

The Low Nuclear Binding Activity Is Characterized by a Reduced Expression of IκBα and of NF-κB-luciferase Reporter Gene—The low IκBα protein levels in the 1.5 and 5 Zn cells could be the result in part of an increased degradation of the inhibitory peptide, secondary to NF-κB activation. However, because the transcription of the IκBα gene is controlled by NF-κB, a decrease in NF-κB-nuclear DNA binding could also lead to a decreased transactivation of the gene. As evaluated by Northern blot (Fig. 6*A*), IκBα mRNA levels were lower in the 1.5 and 5 Zn cells than in the other groups. Expression of the results as the ratio IκBα/28 S mRNAs shows a 50% reduction in IκBα expression in the low zinc cells.

The influence of variations in intracellular zinc on NF-κB-driven transactivating activity in IMR-32 cells was next tested using a reporter gene assay. Cells were co-transfected with a

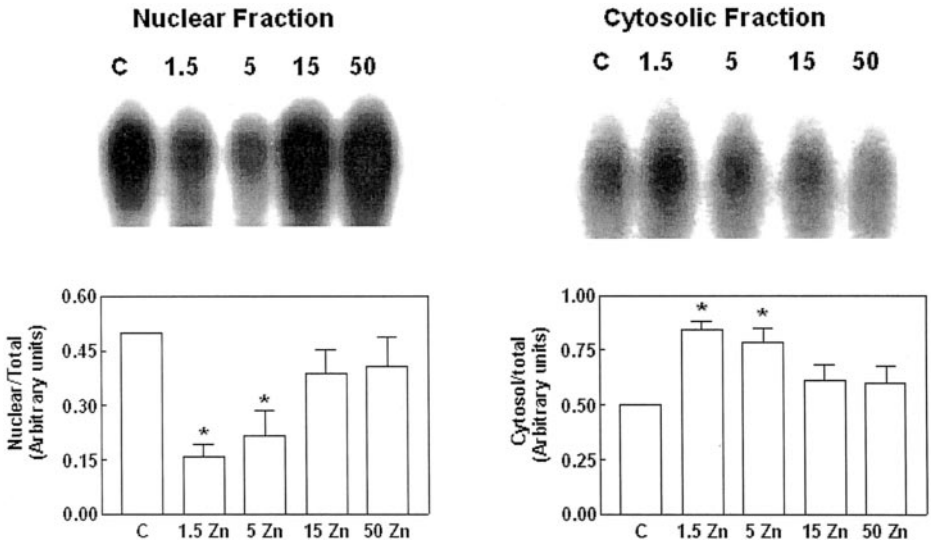

FIG. 4. **NF-κB DNA binding activity in nuclear and cytosolic fractions from IMR-32 cells.** Fractions were isolated after 24 h of exposure to control nonchelated media (*C*) or to chelated media containing 1.5 (*1.5 Zn*), 5 (*5 Zn*), 15 (*15 Zn*), or 50 (*50 Zn*) μM zinc. After the EMSA assays, the bands were quantitated as described under "Experimental Procedures," and values are presented as the ratio of arbitrary units in the cytosol or the nuclei/total (nuclei + cytosol). Results are shown as means ± S.E. of eight independent experiments. *, significantly different compared with control, 15 Zn, and 50 Zn groups ($p < 0.001$, one-way ANOVA test).

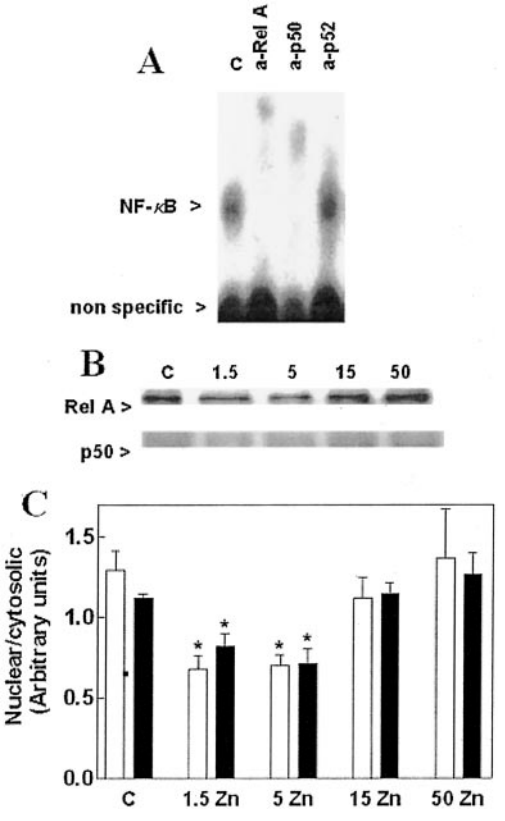

FIG. 5. **Evaluation of possible alterations in the translocation of NF-κB measured by Western blot.** *A*, characterization of the components of the activated NF-κB by an EMSA supershift assay. A control (*C*) nuclear fraction was incubated in the presence of antibodies against RelA, p50, or p52, prior to the binding assay. *B*, Western blot for RelA and p50 of nuclear fractions. *C*, after quantitation of Western blots, results for RelA (*empty bars*) and p50 (*full bars*) are expressed as the ratio nuclear/cytosolic content. Results are shown as means ± S.E. of five independent experiments. *, significantly lower compared with control, 15 Zn, and 50 Zn groups ($p < 0.001$, one-way ANOVA test).

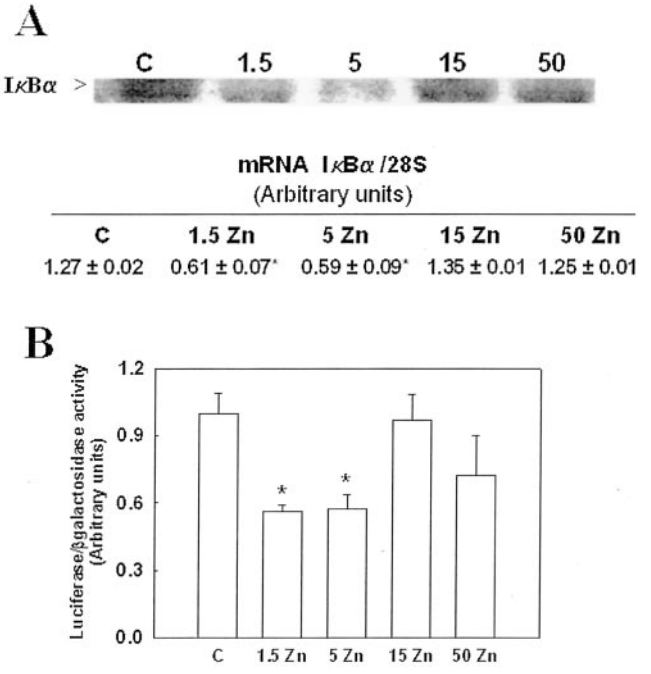

FIG. 6. **Low extracellular zinc concentrations inhibit NF-κB-driven transactivating activity after 24 h of incubation.** *A*, IκBα mRNA levels were measured by Northern blot. After quantitation, results were expressed as the ratio of IκBα/28 S mRNAs. *B*, transactivation of pNF-κB-Luc plasmid. Data are expressed as the ratio luciferase/β-galactosidase activity. Results are shown as means ± S.E. of four independent experiments. *, significantly lower compared with control and 15 Zn groups ($p < 0.001$, one-way ANOVA test).

vector expressing β-galactosidase (as a control of the transfection efficiency) and pNF-κB-Luc plasmid. After 24 h of incubation in the different media, luciferase activity, corrected for β-galactosidase activity, was ~40% lower in the 1.5 and 5 Zn cells than in the control and 15 Zn groups (Fig. 6*B*).

Low Intracellular Zinc Concentrations Are Associated with an Impaired Tubulin Polymerization—The observation that alterations in tubulin polymerization can affect NF-κB activation and translocation (11), combined with reports of impaired tubulin polymerization in brain extracts obtained from zinc-deficient animals (11–14), led us to test the influence of varying extracellular zinc on tubulin polymerization in IMR-32 cells. Fig. 7*A* depicts the typical kinetics of tubulin polymerization in supernatants from control and 5 Zn cells. The rates of tubulin assembly were markedly lower in the 1.5 and 5 Zn (74 and 80%, respectively) cells than in control cells ($p < 0.001$). The tubulin polymerization rate in the 15 and 50 Zn cells was similar to values obtained for the control cells. Prior to the polymerization reaction, total protein (data not shown) and β-tubulin (Fig. 7*B*) concentrations in the 100,000 × *g* supernatants were similar among the groups.

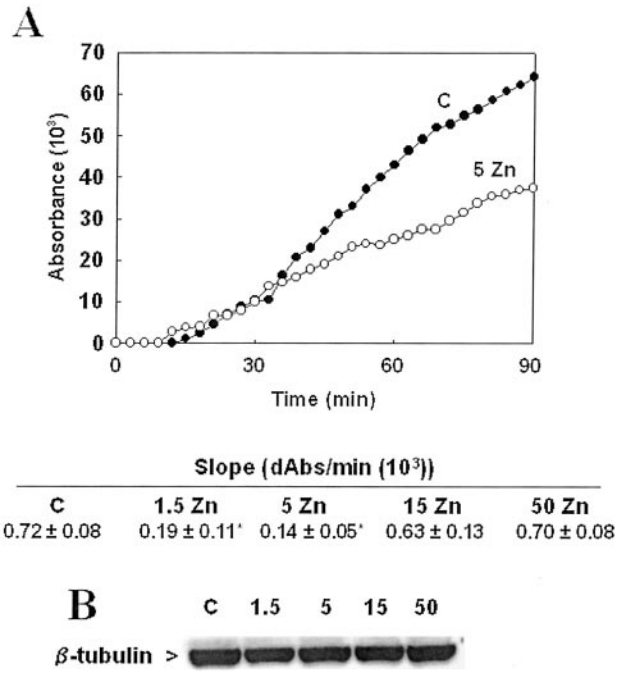

FIG. 7. **Low extracellular zinc concentrations inhibit tubulin polymerization.** Cells were incubated for 24 h in control nonchelated media (*C*) or in chelated media containing 1.5 (*1.5 Zn*), 5 (*5 Zn*), 15 (*15 Zn*), or 50 (*50 Zn*) μM zinc. *A*, tubulin polymerization kinetics for control and 5 Zn cells. The slope in the linear portion of the curves was calculated, and results are shown as means ± S.E. of five independent experiments. *, significantly lower compared with control, 15 Zn, and 50 Zn groups ($p < 0.001$, one-way ANOVA test). *B*, β-tubulin concentration evaluated by Western blot in the 100,000 × *g* supernatants, before the induction of the polymerization reaction.

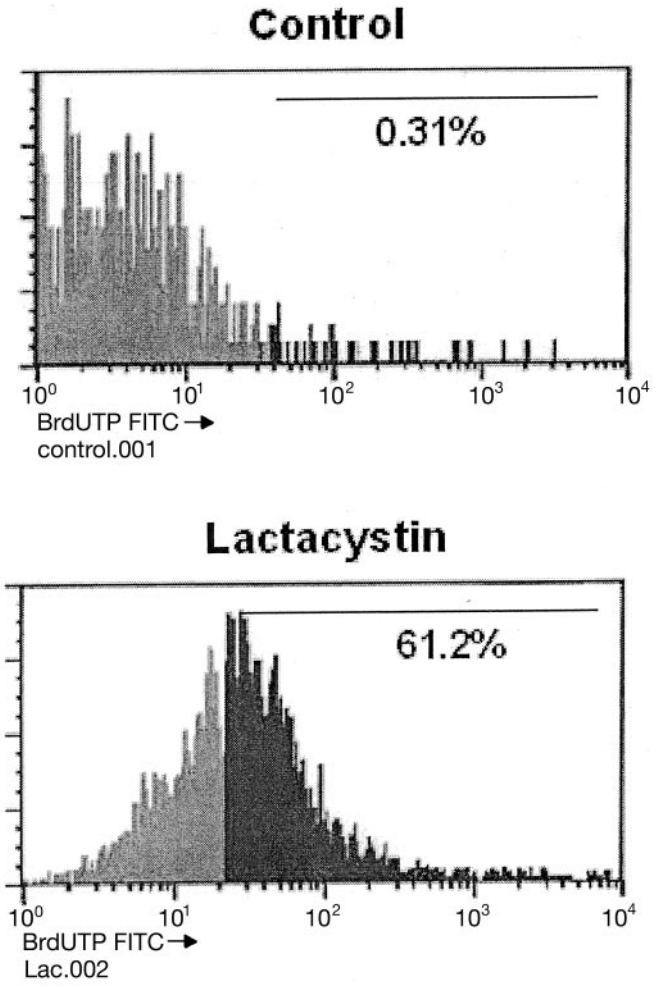

FIG. 8. **Lactacystin, an inhibitor of the proteasome, induces cell death by apoptosis in human neuroblastoma IMR-32 cells.** Cells were incubated for 24 h in control nonchelated media either in the absence (*Control*) or the presence (*Lactacystin*) of 10 μM lactacystin. Cells undergoing apoptosis were evaluated by measuring DNA strand breaks through the initial labeling with bromolated deoxyuridine triphosphates, the secondary reaction with fluorescein-labeled anti-bromodeoxyuridine antibody and subsequent analysis by flow cytometry. The numbers indicate positive cells in percentages. One example of three independent analyses is shown.

In IMR-32 Cells, Inhibition of the Proteasome Leads to Apoptosis—Although NF-κB in general mediates signals of cell survival, in certain cell types it can exert a pro-apoptotic action. To assess whether the inhibition of NF-κB in human neuroblastoma IMR-32 cells can lead to apoptosis, cells were treated with lactacystin, an inhibitor of the proteasome. DNA fragmentation, a late event in apoptosis, was measured after 24 h of incubating cells in control media with, or without, the addition of 10 μM lactacystin. The DNA breaks were measured by incorporating bromolated deoxyuridine triphosphates in a reaction catalyzed by the terminal deoxynucleotidyltransferase enzyme. Cell cytometry analysis of the samples showed that control cells had nondetectable apoptotic events, whereas, in those treated with lactacystin, 61.9 ± 0.7% of the cells were apoptotic (Fig. 8). No propidium iodide-positive cells were observed in both cell populations (data not shown).

DISCUSSION

In the present study, we investigated the modulatory effects of zinc on the oxidant-responsive transcription factor NF-κB, with a focus on the steps in the activation cascade that could be affected by low intracellular zinc in a human neuroblastoma cell line (IMR-32).

We previously reported that zinc deficiency results in reduced NF-κB nuclear binding activity in 3T3 fibroblasts (9), as well as in testes obtained from zinc-deficient rats (10). These findings were unexpected, as we and other investigators have demonstrated that a low zinc status is associated to a condition of oxidative stress (9, 10, 25–34). NF-κB is recognized to be activated by different oxidant species, by conditions that induce the intracellular production of oxidants and by oxidative-degradation products (35–38). In zinc deficiency, the imbalance between the enzymes that generate and metabolize H_2O_2 (9, 34) can result in a rapid increase of H_2O_2. Consistent with this, we have measured a marked increase in H_2O_2 production in IMR-32 cells exposed to media containing 1.5 or 5 μM zinc (39).

TSQ-reactive intracellular zinc decreased in the cells incubated in low zinc media (1.5 and 5 Zn) after only 3 h of exposure; TSQ-reactive intracellular zinc levels were similar in cells supplemented with 15 or 50 μM zinc, and in cells incubated in control nonchelated media. TSQ has been used to measure zinc after cerebral ischemia (40). Dithizone, a cell-permeable and zinc-specific chelator, abolished TSQ fluorescence, indicating that TSQ binds to intracellular zinc. Because TSQ fluorescence depends on the polarity of the solvent, and binds preferentially to membrane zinc (41), the obtained results cannot be extrapolated to absolute values. Total zinc concentrations were lower in the 1.5 and 5 Zn cells compared with the control cells, and cells incubated in media containing 15 and 50 μM zinc.

Although the number of viable cells was similar in the groups after 12 h in culture, by 24 h, the number of viable cells was significantly lower in the 1.5 and 5 Zn groups than in the other three groups. This effect could be the result of an arrest in cell division, given the requirements for zinc at multiple steps of the cell cycle, or the result of an increased rate of cell

death in the 1.5 and 5 Zn groups.

We investigated the total level of NF-κB activation both by EMSA of the total fractions and by the phosphorylation and concentration of IκBα. We observed a high level of NF-κB binding activity in the groups with low concentrations of intracellular TSQ-reactive zinc (1.5 and 5 Zn). This effect could be the result of the higher H_2O_2 production found in these cells (39), an important signal in the activation of NF-κB (6, 36–38, 42). The inhibitory peptide IκBα (one member of the inhibitory IκB proteins) binds to NF-κB and prevents the translocation of the active dimer to the nuclei and its binding to DNA. During NF-κB activation, IκBα is phosphorylated by the serine-specific IκB kinase, with posterior ubiquitinization and degradation of IκBα by the proteasome (8). We observed a high level of IκBα phosphorylation at 24 h, and a low content of IκBα at 24 and 48 h, in the 1.5 and 5 Zn groups, compared with the other three groups. These results are in agreement with those obtained with the EMSA assay for total cell fractions. We conclude that low intracellular zinc concentrations trigger cytosolic events involved in NF-κB activation.

A low NF-κB nuclear binding activity was found in the 1.5 and 5 Zn groups. However, when the binding activity was measured in the cytosolic fractions, we observed significantly higher levels in the 1.5 and 5 Zn cells than in the other groups. These results indicate that the low nuclear binding of NF-κB that we have observed in different cell types is not related to a low level of NF-κB activation, but rather to alterations in the translocation of the active transcription factor from the cytosol to the nuclei.

The activated NF-κB in IMR-32 cells is composed by p50 and RelA proteins. As determined by Western blot, the concentrations of RelA and p50 were high in the cytosol and low in the nuclei; the ratio of nuclear/cytosolic content was ~45% lower in the 1.5 Zn and 5 Zn cells than in the other three groups. These findings reinforce the EMSA finding and support the concept that there is an alteration in the translocation of the active NF-κB to the nuclei in the cells with low intracellular zinc.

The MAD-3 gene contains κB sites in its promoter, and its transcription is regulated by different combinations of the Rel/NF-κB family proteins (43). Thus, the low IκBα levels observed in the 1.5 and 5 Zn groups could be, in part, caused by the activation of upstream events leading to IκBα degradation, as well as to a lower transactivating activity by NF-κB, with a consequent lower expression of IκBα. In the 1.5 and 5 Zn cells, we observed a 50% reduction in the IκBα mRNA levels, demonstrating that the low nuclear NF-κB binding activity results in an impaired transactivation of NF-κB-driven genes. Consistent with this observation, cells transiently transfected with a plasmid containing a luciferase gene linked to an enhancer containing five NF-κB sites had lower luciferase activity when they were incubated in 1.5 and 5 μM zinc than that observed when they were incubated in the other media.

Experimental evidence supports the concept that alterations in the cytoskeleton, as well as in cell shape, can affect transcription factors, such as NF-κB, that exist in the cytosol in an inactive form and that, after their activation, are translocated to the nuclei. The state of tubulin polymerization seems to be crucial in NF-κB nuclear translocation, as well as in the subsequent expression of target genes. In the promoter of the proto-oncogene c-*myc* there are two κB sites (44) indicating that NF-κB can regulate c-*myc*. In human colon adenocarcinoma cells, vinblastine and nocodonazole, both inhibitors of tubulin polymerization, lead to NF-κB activation (45). The vinblastine-induced transactivation of c-*myc* is partially dependent on NF-κB as mutations in the κB sites decrease the capacity of vinblastine to stimulate the transactivation of c-*myc*. However, the influence of agents that affected microtubules on c-*myc* induction depended on the cell line (46).

Tubulin depolymerization, induced by pharmacological inhibitors such as nocodazole, or by cold, can activate NF-κB leading to IκBα degradation (11). In earlier work, we reported that zinc deficiency can alter the kinetics of brain tubulin polymerization in rats, a lower initial velocity and a longer lag period in tubulin assembly being observed in brain supernatants obtained from zinc-deficient rats compared with zinc-supplemented controls (12–14). Consistent with the above, IMR-32 cells, with low intracellular zinc concentrations (1.5 and 5 μM extracellular zinc), at similar β-tubulin content, had low rates of tubulin polymerization. This observation indicates that low intracellular zinc concentrations reduce the rate of tubulin polymerization. We suggest that the above represents one signal for NF-κB activation in the zinc-deficient cell. Although tubulin depolymerization can trigger NF-κB activation, intact microtubules are needed to subsequently transport NF-κB into the nucleus. We propose that this second step is impaired in the zinc-deficient cell, which results in a building of active NF-κB in the cytosol. Similar to our findings, cold-induced NF-κB activation in HeLa S3 cells was associated with the accumulation of the active dimer in the cytosol, which was reverted by warming the cells at 37 °C (11). Immunoblot analysis (using an anti-β-tubulin antibody) of dimeric and polymeric tubulin confirmed the depolymerization of microtubules after incubation at 4 °C and a fast repolymerization upon warming at 37 °C (11).

In neurons, the anti-apoptotic action of NF-κB has been proposed to be mediated through the regulation of NF-κB-driven genes such as *Bcl*II, manganese superoxide dismutase, and proteins involved in calcium homeostasis (47). Several other genes regulated by NF-κB, such as those for cyclin D1, Blf-11, Bcl-xl, caspase inhibitors, TRAF1, and TRAF2, may also be involved in the protection of cells against apoptosis (see Ref. 17 for a review). Although in general NF-κB acts as a survival signal, in some cell types, and depending on the experimental condition, NF-κB can exert an opposite effect and trigger apoptosis cascades. We studied whether, in human neuroblastoma IMR-32 cells, the inhibition of NF-κB induces apoptosis. After treatment for 24 h with 10 μM lactacystin, a specific inhibitor of the proteasome, 62% of the cells were apoptotic. Given that the proteasome participates in the activation of NF-κB, through the degradation of the polyubiquitylated IκBα secondary to IκBα phosphorylation, the above work with lactacystin supports the concept that in the IMR-32 cell line NF-κB acts as a survival signal, inhibiting cell apoptosis.

In summary, we have demonstrated that low intracellular zinc concentrations can trigger the activation of the early stage in NF-κB activation. The above may be the result of zinc deficiency-induced reductions in microtubule polymerization, increases in intracellular oxidants, and an altered thiol redox state. An impairment in microtubule assembly can result in a reduction in the translocation of the active transcription factor to the nuclei, thus impairing the transactivation of genes involved in apoptosis. If the above finding with the IMR-32 cell line can be documented in the developing brain, it would suggest that an impairment in microtubule polymerization that in turn alters the NF-κB signaling cascade could be a mechanism contributing to the brain defects associated with developmental zinc deficiency. Alterations in the decisions of neuronal cells to proliferate, differentiate, or undergo apoptosis could contribute to the occurrence of the brain defects associated with zinc deficiency.

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