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α-Lipoic acid and *N*-acetyl cysteine prevent zinc deficiency-induced activation of NF-κB and AP-1 transcription factors in human neuroblastoma IMR-32 cells

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Abstract
This work investigated the capacity of α-lipoic acid (LA) and *N*-acetyl-L-cysteine (NAC) to reduce zinc deficiency-induced oxidative stress, and prevent the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), and the cross-talk between both activated cascades through β-Transducin Repeat-containing Protein (β-TrCP). IMR-32 cells were incubated in control media or media containing variable concentrations of zinc, without or with 0.5 mM LA or 1 mM NAC. Relative to control and zinc supplemented (15 μM Zn) groups, Hydrogen peroxide (H_2O_2) and total oxidant cell concentrations were higher, and total glutathione concentrations were lower in the zinc deficient groups (1.5 and 5 μM Zn). Both, LA and NAC, markedly reduced the increase in cell oxidants and the reduction in glutathione concentrations in the zinc deficient cells. Consistent with this, LA and NAC prevented zinc deficiency-induced activation of the early steps of NF- κB (IκBα phosphorylation) and AP-1 [c-Jun-N-terminal kinase (JNK) and p38 phophorylation] cascades, and the high NF-κB- and AP-1-DNA binding activities in total cell extracts. Thus, LA and NAC can reduce the oxidative stress associated with zinc deficiency and the subsequent triggering of NF-κB- and AP-1-activation in neuronal cells.

Keywords: *Zinc, NF-κB, AP-1, lipoic acid,* N*-acetyl-cysteine*

Abbreviations: *LA, (±)-α-lipoic acid; NAC,* N*-acetyl cysteine; AP-1, activator protein-1; NF-κB, nuclear factor-κB; JNK, c-Jun-N-terminal kinase; MAPKs, mitogenactivated protein kinases; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; EDTA, ethylenediamine tetraacetate; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; β-TrCP, β-Transducin repeat-containing protein; DTPA, diethylenetriamine pentaacetic acid; PBS, phosphate-buffered saline; TSQ,* N*-6-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide; DCDHF, 5(or 6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate; DCF, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein; EMSA, electrophoretic mobility shift assay*

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Introduction

Zinc can function as an antioxidant at multiple levels [1,2], and zinc deficiency can be associated with an increase in oxidative stress [3–6]. In animal and cell models, zinc deficiency can result in an increase in cell oxidant concentrations [4,7], alterations of the oxidant defense system [4,6,8] and oxidative damage to proteins, lipids and DNA [5,7].

The NF-κB family of transcription factors is formed by homodimers or heterodimers of Rel/NF-κB proteins that are involved in the regulation of numerous genes, including those related to cell proliferation and apoptosis [9]. The NF-κB can be activated by different oxidant species, by changes in the thiol redox status, by conditions that increase the intracellular levels of oxidants and by oxidative-degradation products [10–13].

Antioxidants can modulate NF-κB activation [14–17], in some cases through mechanisms distinct from redox regulation [18,19]. Reactive oxygen species are thought to activate NF-κB through the phosphorylation of the inhibitory protein IκB at Ser32 and 36, which marks IκB for further ubiquitination and degradation by the proteasome [20]. The active NF-κB subsequently translocates to the nuclei where it regulates gene expression.

AP-1 is also a redox-sensitive transcription factor [21,22]. AP-1 transcription factors consist of homo or heterodimers formed by different combinations among the Jun, Fos, Maf and ATF subfamilies of proteins [23]. The activation of AP-1 depends mainly on its induction and phosphorylation by the mitogen-activated protein kinases (MAPKs) [24]. Two main MAPK signaling pathways, c-Jun-N-terminal kinase (JNK) and p38, are sensitive to oxidative stress, and both can be activated by hydrogen peroxide (H_2O_2) [22], or by an altered glutathione redox status [25]. In the central nervous system, AP-1 can be activated by select reactive oxygen species, including superoxide anion and H_2O_2 [26,27] and this activation can be blunted by select antioxidants (See Finkel and Holbrook for a review [28]).

Glutathione is one of the main components of the oxidant defense system and it can play a major role in the regulation of gene expression [29]. (±)-α-lipoic acid (LA) and *N*-acetyl cysteine (NAC) are antioxidants involved in the maintenance of cellular thiols. The LA has multiple described mechanisms through which it can exert its antioxidant action. The LA can directly scavenge oxidant species, chelate redox-active metals and reduce glutathione [15,30,31]. NAC is a source of cysteine, a precursor in the synthesis of glutathione, and it can play a role as an antioxidant by increasing the intracellular pools of thiols for free radical scavenging or by interacting with reactive oxygen or reactive nitrogen species [32,33].

Zinc deficiency is often characterized by increased levels of reactive oxygen and nitrogen species [4,7], and consistent with the involvement of oxidants in the activation of NF-κB and AP-1, the activation of both transcription factors can be increased with zinc deficiency [4,34]. In human neuroblastoma IMR-32 cells, a decrease in intracellular zinc concentrations can lead to the activation of the cytosolic events of the NF-κB activation cascade, increasing the phosphorylation and degradation of the inhibitory peptide IκBα, resulting in high levels of NF-κB-DNA binding activity in total cell fractions [35]. Similarly, in both 3T3 and IMR-32 cells, zinc deficiency can result in high levels of AP-1-DNA nuclear binding activity, presumably secondary to oxidative stress [4].

In the present study, we investigated if, in human neuroblastoma IMR-32 cells, LA and NAC could prevent zinc-deficiency-induced changes in intracellular oxidants, and the subsequent activation of NF-κB and AP-1 transcription factors. Possible cross-talk between the cascades through β-Transducin repeat-containing protein (β-TrCP) was also studied.

Materials and methods

Materials

IMR-32 cells were obtained from the American Type Culture Collection (Rockville, MA). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlbad, CA). The CellTiter 96® non-radioactive cell proliferation assay, the oligonucleotides containing the consensus sequences for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGAGTCAGCC-GGAA-3′) and the reagents for the electrophoretic mobility shift assay (EMSA) were obtained from Promega (Madison, WI). Antibodies for phospho-JNK (p-JNK), JNK, IκBα and β-TrCP were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-p38 (p-p38) and phospho-IκBα (p-IκBα) were from Cell Signaling Technology (Beverly, MA). PVDF membranes were obtained from BIO-RAD (Hercules, CA) and Chroma Spin-10 columns from Clontech (Palo Alto, CA). *N*-6-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ), propidium iodide and 5(or 6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (DCDHF) were obtained from Molecular Probes (Eugene, OR). The ECL western blotting system was from GE Healthcare (formerly Amersham Pharmacia Biotech Inc.) (Piscataway, NJ). LA, NAC and all the other reagents were from the highest quality available and were purchased from Sigma (St Louis, MO).

Cell culture and incubation

IMR-32 cells were cultured at 37°C in complex medium: (55% (v/v) Dulbecco's modified eagle

medium (DMEM) high glucose, 30% (v/v) Ham F-12, 5% (v/v) α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics-antimicotic (50 U/ml penicillin, 50 μg/ml streptomycin and 0.125 μg/ml amphotericin B).

Zinc deficient FBS was prepared by chelation with diethylenetriamine pentaacetic acid (DTPA) as previously described [4]. The chelated FBS was subsequently diluted with complex medium to a final concentration of 3 mg protein/ml to match the protein concentration of the control non-chelated media (10% (v/v) FBS). The zinc concentration of the zinc deficient medium was 1.5 μM and portions of this media were supplemented with $ZnCl_2$ to reach concentrations of 5 and 15 μM.

Cells were grown in control medium (complex medium containing 10% non-chelated FBS) until 90% confluence, after which the media was removed and replaced with control medium or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc. In each zinc group, aliquots of cells were also incubated for 24 h in medium that contained 0.5 mM LA or 1 mM NAC.

For the determination of cell viability, cells were cultured in 96 well plates. Cell viability was evaluated by measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) to a formazan product using the CellTiter 96® non-radioactive cell proliferation assay (Promega, Madison, WI) following the manufacturer's protocols.

Determination of intracellular zinc levels

The content of TSQ-reactive zinc was evaluated as previously described [35]. Briefly, cells (1.2×10^6) were incubated in the corresponding media for 24 h, the medium was decanted, cells were rinsed with warm DMEM and 1 ml of DMEM containing 25 μM TSQ was added. Cells were dispersed and incubated at 37°C in the dark for 20 min. Cells were transferred to 1.5-ml conical tubes and centrifuged at 800*g* for 10 min. The cell pellet was rinsed twice with PBS and finally resuspended in 0.2 ml of PBS containing 0.1% (v/v) Igepal. After a brief sonication, the fluorescence at 480 nm (λ_{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 (λ_{exc}: 538, λ_{em}: 590) was measured. Results are expressed as the ratio TSQ fluorescence/propidium iodide fluorescence.

Determination of cell oxidant levels

Cell oxidant levels were evaluated using the probe DCDHF, which can cross the cell membrane, and after oxidation, is converted to 5-(and-6)-carboxy-2′,7′- dichlorofluorescein (DCF), a fluorescent compound. Cells (1×10^5) were grown in 12 well plates. After the corresponding treatments, the media was discarded; cells were rinsed with PBS and suspended in 200 μl of DMEM containing 10 μM DCDHF. After 30 min of incubation at 37°C, the media was removed; cells were rinsed with PBS, and then incubated in 200 μl phosphate-buffered saline (PBS) containing 0.1% (v/v) Igepal. After 30 min of incubation with regular shaking, the fluorescence at 525 nm (λ_{exc}: 475 nm) was measured. To evaluate DNA content, samples were subsequently incubated with 50 μM propidium iodide. After incubating for 20 min at room temperature, the fluorescence (λ_{exc}: 538 nm, λ_{em}: 590 nm) was measured. Results are expressed as the ratio DCF fluorescence/propidium iodide fluorescence.

Determination of H_2O_2 release

H_2O_2 was measured according to Szatrowski et al. [36] using a scopoletin fluorescence assay in which H_2O_2 oxidizes up to 30 μM scopoletin to a non-fluorescent compound in a reaction catalyzed by 1 unit/ml horseradish peroxidase. The presence of 1 mmol/l sodium azide in the reaction mixture prevents the competitive action of catalase on H_2O_2. Cells were cultured in 96 well plate for 24 h under the different treatments, then the media was removed and cells were incubated in the reaction mixture prepared in phenol red-free DMEM for 3 h. Fluorescence was measured using a Perkin-Elmer HTS 7000 Plus Bio Assay Reader (Perkin-Elmer Life Sciences). To evaluate DNA content, samples were subsequently incubated with 50 μM propidium iodide. After incubating for 20 min at room temperature, the fluorescence (λ_{exc}: 538 nm, λ_{em}: 590 nm) was measured. Results are expressed as H_2O_2 concentration normalized to propidium iodide fluorescence.

Determination of total glutathione concentration

Total glutathione was determined by HPLC with electrochemical detection. After the corresponding treatments, the media was discarded; cells (20×10^6) were rinsed with PBS, collected in 50 μl of PBS and frozen at −80°C. After thawing, cells were sonicated for 2 cycles of 20 s at 60 W each. While one aliquot was separated for protein content, the rest of the sample was mixed with 1 M perchloric acid solution containing 2 mM ethylenediamine tetraacetate (EDTA) and centrifuged at 10,000*g* for 10 min to separate the protein precipitate. The supernatant fraction was filtered though a 0.2 μm pore size filter. Samples were frozen at −80°C until analyzed for glutathione concentration. Separation was achieved using a Supelcosil LC-18 (250 × 4.6 mm I.D., 5 μm particle size, 100 Å pore size) reversed-column and a Supel guard (20 × 4.6 mm I.D.), pellicular reversed-phase cartridge precolumn, both purchased from Supelco

(Supelco, Bellofonte, PA, USA). Elution was carried out isocratically with 20 mM sodium phosphate buffer, pH 2.7 as the mobile phase. Chromatographic separation was performed at room temperature at a flow of 1.5 ml/min. The potential setting of the Couluchem II multidetector was: guard cell, +0.900 V; detector 1, + 0.450 V; and detector 2, + 0.800V. Results are expressed as pmol of total glutathione/μg protein.

Electrophoretic mobility shift assay (EMSA)

Total cell fractions were prepared as previously described [37]. After 24 h under the different experimental conditions, the media was discarded, cells were rinsed with PBS and scraped. After centrifugation at 800*g* for 10 min at 4°C, the pellet (3×10^6 cells) was lysed in 100 μl of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.9, containing 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1% (v/v) Igepal, 20% (v/v) glycerol, 5 mM dithiothreitol (DTT) and EDTA-free protease inhibitors (Roche Applied Science, IN) and vortexed. Samples were kept on ice for 30 min and then cleared by centrifugation at 10,000*g* for 15 min at 4°C. Protein concentration was determined by the method of Bradford [38] and samples were stored at −80°C.

For the EMSA, the oligonucleotides containing the consensus sequences for NF-κB or AP-1 were 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 4% (v/v) glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl and 0.05 mg/ml poly(dI − dC). The products were separated by electrophoresis in a 4% (w/v) non-denaturing polyacrilamide 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 Phosphoimager 840 (GE Healthcare, Piscataway, NJ).

Western blot analysis

Total cell extracts (3×10^6 cells) were prepared for the evaluation of β-TrCP and IκBα as previously described [35]. To evaluate the phophorylation of IκBα, p38 and JNK, cell lysates were prepared in SDS-sample buffer (62.5 mM Tris–HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT and 0.01% (w/v) bromophenol blue), sonicated for 15 s to shear DNA and reduce sample viscosity, and then heated at 95°C for 5 min. Immediately, 30 μl of the lysate were loaded onto SDS-PAGE. Proteins were separated by reducing 10–12.5% (w/v) polyacrilamide gel electrophoresis and electroblotted to PVDF membranes. Colored molecular weight standards (GE Healthcare, Piscataway, NJ) were ran simultaneously. Membranes were blocked overnight in 5% (w/v) non-fat milk and incubated in the presence of the corresponding primary antibodies (1:1000 dilution) for 90 min at 37°C. After incubation for 90 min at room temperature in the presence of the secondary antibody (HRP-conjugated) (1:10,000 dilution) the conjugates were visualized by chemiluminescence's detection in a Phosphoimager 840. The membranes were normalized by reblotting with the antibody for the corresponding non-phosphorylated form of each protein, or for β-tubulin when β-TrCP was assessed

Statistical analysis

One way analysis of variance (ANOVA) with subsequent *post hoc* comparisons by Scheffe was performed using Statview 5.0.1 (Brainpower Inc., Calabazas, CA). A p value < 0.05 was considered statistically significant. Values are given as means ± SEM.

Results

LA and NAC prevent the increase in cellular oxidant levels and of H_2O_2, and the decrease in total glutathione in zinc deficient cells

To assess the induction of a condition of zinc deficiency by incubating IMR-32 cells in media with low zinc concentration, the TSQ probe was used. TSQ can cross membranes and react with membrane- and loosely-bound zinc. Both zinc pools are considered to be rapidly available for cellular requirements. After 24 h of incubation, cellular zinc was significantly lower ($p < 0.02$, one way ANOVA test) in the 1.5 and 5 μM zinc cells than in control and 15 μM zinc groups (Figure 1). This decrease in cellular zinc was not prevented by the simultaneous treatment with either 0.5 mM LA or 1 mM NAC.

After 24 h of incubation, a decrease in the number of viable cells in the 1.5 and 5 μM zinc groups (38 and 32%, respectively) was observed. The number of viable cells war similar between C and 15 μM zinc groups. The simultaneous treatment of zinc deficient cells (1.5 and 5 μM zinc) with either 0.5 mM LA or 1 mM NAC prevented the decrease in cell viability.

Incubation in zinc deficient media (1.5 or 5 μM zinc) led to a 66 and 48% increase in cell oxidant levels in the 1.5 and 5 μM zinc cells, respectively, compared to the control and 15 μM zinc groups. This increase was not observed when cells were simultaneously incubated with LA or NAC (Figure 2A). Consistent with the above, treatment with LA or NAC inhibited the increased release of H_2O_2 in the zinc deficient cells (Figure 2B). The cellular oxidant levels and H_2O_2

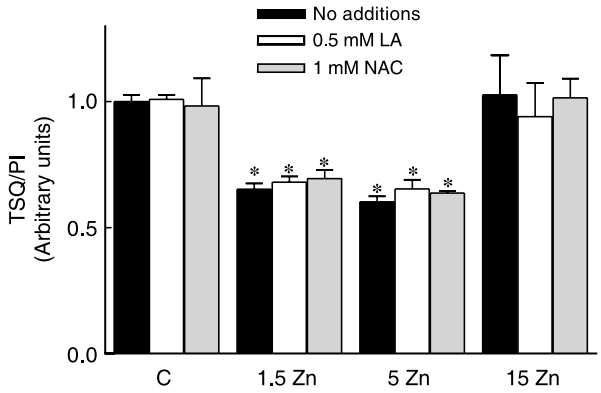

Figure 1. Intracellular TSQ-reactive zinc levels. TSQ-reactive zinc was measured in cells incubated for 24 h in control non-chelated media (C) or in chelated media containing 1.5, 5 or 15 μM zinc, incubated simultaneously without (full bars) or with either 0.5 mM LA (empty bars) or 1 mM NAC (grey bars). The intracellular zinc concentration was determined as described in "Materials and Methods" section. TSQ fluorescence was normalized to the propidium iodide fluorescence (TSQ/PI) in each sample to correct for differences in cell number. Values are shown as the means ± SEM of 3 independen experiments. *Significantly different compared to all other tested conditions ($p < 0.02$, one way ANOVA test).

release values were similar for the control and 15 μM zinc groups, and were not significantly affected by LA and NAC treatment.

Glutathione, the main free non-protein thiol of cells, is a critical neuronal defense against an increase in cell oxidants. After 24 h, a 24 and 33% decrease in total glutathione concentrations was observed in the 1.5 and 5 μM zinc cells, respectively, compared to the control and 15 μM zinc groups (Figure 2C). While treatment with LA or NAC did not affect glutathione values in the control or 15 μM zinc groups, it prevented the reduction of glutathione in the zinc deficient cells (Figure 2C).

LA and NAC prevented NF-κB activation in zinc deficient cells

Figure 3A depicts the EMSA for NF-κB in total cell fractions. After 24 h, the DNA binding activity of NF-κB in total cell fractions was approximately 80% higher in the 1.5 and 5 μM Zn cells, than in the control and 15 μM zinc groups (Figure 3B). Incubation with either LA or NAC prevented the increase in NF-κB-DNA binding activity associated with zinc deficiency (Figure 3A and B). An upstream event in the NF-κB activation cascade was characterized by measuring the phosphorylation of the inhibitory peptide IκBα. In agreement with the EMSA observations, the ratio of phosphorylated IκBα/IκBα was 2-fold higher in the 1.5 and 5 μM zinc cells than in the control and 15 μM zinc groups (Figure 4B). The incubation of the zinc deficient cells with LA or NAC prevented the increase in IκBα phosphorylation

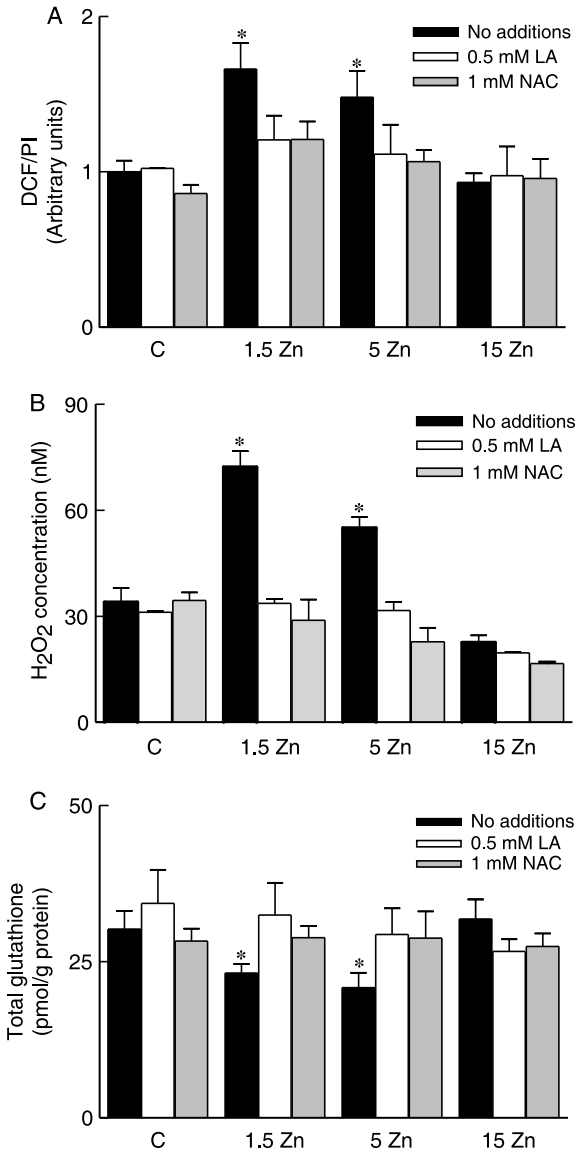

Figure 2. LA and NAC prevented zinc deficiency-induced increase in cellular oxidant levels and H_2O_2 release, and the decrease in total glutathione concentration. (A) Cellular oxidant levels were measured in IMR-32 cells after 24 h of culturing cells in control non-chelated media (C) or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, incubated simultaneously without (full bars) or with either 0.5 mM LA (empty bars) or 1 mM NAC (grey bars). Cellular oxidant levels were determined as described under "Materials and Methods" section. DCF fluorescence was normalized to the propidium iodide (PI) fluorescence. (B) H_2O_2 release to the media was determined fluorometrically by incubating cells with 30 μM scopoletin for 3 h. Cells had been previously incubated for 24 h in control non-chelated media (C) or in chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, without (full bars) or with either 0.5 mM LA (empty bars) or 1 mM NAC (grey bars). H_2O_2 concentrations (nM) were normalized to propidium iodide (PI) fluorescence. (C) Total glutathione concentrations were evaluated by HPLC as described under "Materials and Methods" section, in total cell fractions after 24 h of culturing cells in the different experimental conditions. Glutathione content was normalized by the protein content. Results are shown as means ± SEM of 4 independent experiments. *Significantly different compared to all other tested conditions ($p < 0.01$, one way ANOVA test).

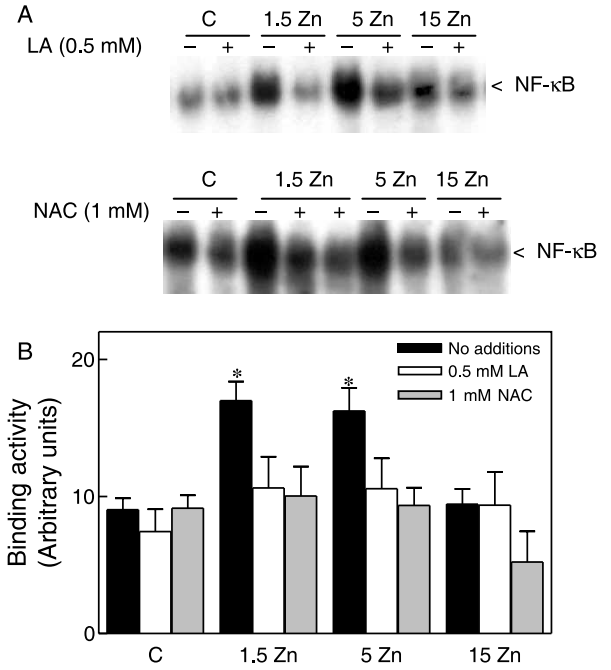

Figure 3. LA and NAC prevented zinc deficiency-induced increase in NF-κBDNA binding activity. Total cell fractions were isolated after 24 h of culturing cells in control non-chelated media (C) or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, incubated simultaneously without or with either 0.5 mM LA or 1 mM NAC. (A) EMSA for NF-κB after 24 h incubation in the corresponding media, (B) after the EMSA assays, bands were quantitated and results correspond to cells incubated without (full bars) or with the addition of LA (empty bars) or NAC (grey bars). Results are shown as means ± SEM of 6 independent experiments. *Significantly different compared to all other tested conditions ($p < 0.001$, one way ANOVA test).

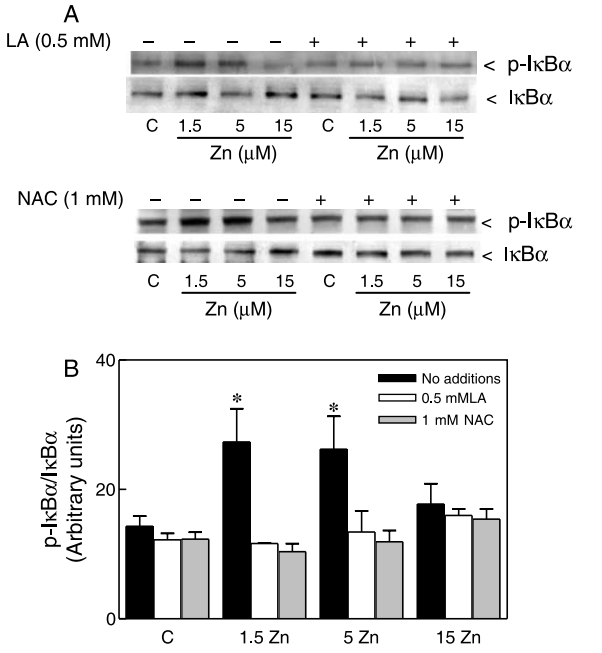

Figure 4. LA and NAC prevented zinc deficiency-induced increase in IκBα phosphorylation. Total cell fractions were isolated after 24 h of culturing cells in control non-chelated media (C) or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, incubated simultaneously without or with either 0.5 mM LA or 1 mM NAC. (A) Western blots for phosphorylated IκBα (p-IκBα) and IκBα, after the corresponding treatments. (B) After quantitation, the ratio phosphorylated IκBα (pIκBα)/IκBα was calculated for cells incubated without (full bars) or with the addition of LA (empty bars) or NAC (grey bars). Results are shown as means ± SEM of 4 independent experiments. *Significantly different compared to all other tested conditions ($p < 0.01$, one way ANOVA test).

(Figure 4A and B). LA and NAC did not affect NF-κB-DNA binding activity or IκBα phosphorylation values, in the control and 15 μM zinc groups.

LA and NAC prevented AP-1 and MAPK activation and increased β-TrCP levels in zinc deficient cells

Similar to NF-κB, after 24 h of incubation, the DNA binding activity of AP-1 in total cell fractions was markedly higher in the 1.5 and 5 μM zinc cells than in control and 15 μM zinc cells (Figure 5A). The increased AP-1 nuclear binding activity observed in the zinc deficient cells was prevented when the cells were incubated with either LA or NAC (Figure 5A and B).

p38 and JNK are stress-sensitive MAPKs involved in the activation of AP-1 by H_2O_2.[24] The effects of LA and NAC on the phosphorylation of the upstream MAPKs, p38 and JNK were measured. After 24 h, higher levels of p38 (Figure 6A) and JNK (Figure 7A) phosphorylation were observed in the 1.5 and 5 μM zinc groups, compared to the control and 15 μM zinc groups. The simultaneous incubation with LA or NAC prevented the increased p38 and JNK phosphorylation associated with zinc deficiency (Figures 6 and 7).

The β-TrCP can activate NF-κB, participating in the proteasome-mediated degradation of IκB. Previous findings indicate that JNK can activate NF-κB by stabilizing β-TrCP mRNA levels [39]. The β-TrCP levels were measured by Western blot in total cell fractions after incubating cells in media with variable zinc concentrations without, or with, LA or NAC. After 24 h of incubation, there was an increase in β-TrCP levels in the 1.5 and 5 μM zinc cells, compared to the control and 15 μM zinc groups (Figure 8). An increase in β-TrCP levels was not observed in the zinc deficient cells cultured with either LA or NAC (Figure 8).

Discussion

Zinc deficiency can induce a condition of oxidative stress, which may be due either to the capacity of zinc to act as an antioxidant [2] or to secondary changes in cell metabolism and function that occur as a consequence of such deficiency. An early consequence of a decrease in cellular zinc is an increase in the steady

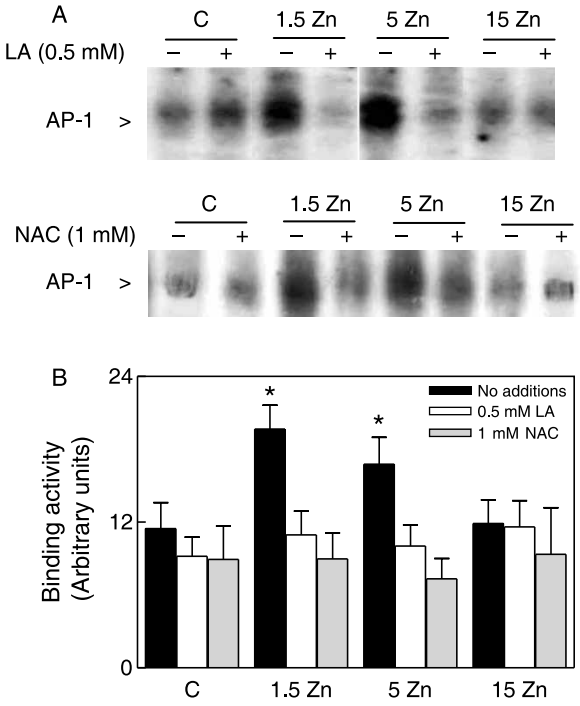

Figure 5. LA and NAC prevented zinc deficiency-induced increase in AP-1-DNA binding activity. Total cell fractions were isolated after 24 h of culturing cells in control non-chelated media (C) or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, incubated simultaneously without or with either 0.5 mM LA or 1 mM NAC. (A) EMSA for AP-1 after 24 h incubation in the corresponding media, (B) after the EMSA assays, bands were quantitated and results correspond to cells incubated without (full bars) or with the addition of LA (empty bars) or NAC (gray bars). Results are shown as means ± SEM of 5 independent experiments. *Significantly different compared to all other tested conditions ($p < 0.05$, one way ANOVA test).

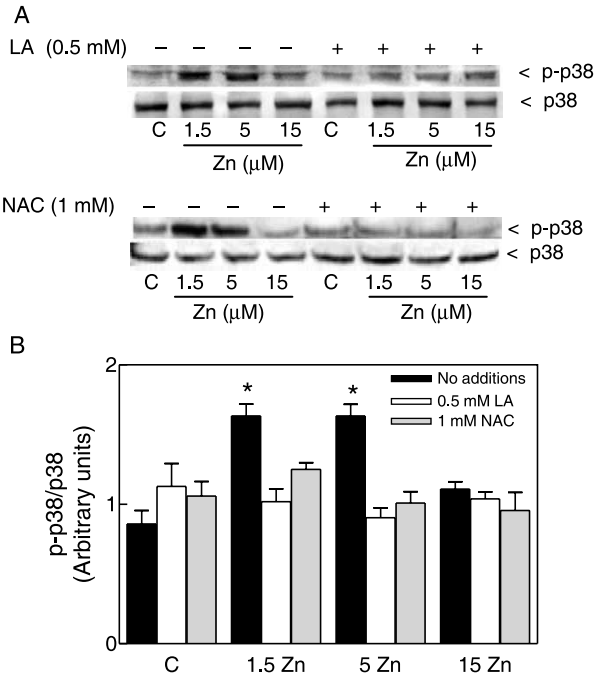

Figure 6. LA and NAC prevented zinc deficiency-induced increase in p38 phosphorylation. Total cell fractions were isolated after 24 h of culturing cells in control non-chelated media (C) or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, incubated simultaneously without or with either 0.5 mM LA or 1 mM NAC. (A) Western blots for phosphorylated p38 (p-p38) and p38, after 24 h of incubation under the different treatments. (B) After quantitation, the ratio p-p38/p38 was calculated for cells incubated without (full bars) or with the addition of LA (empty bars) or NAC (gray bars). Results are shown as means ± SEM of 4 independent experiments. *Significantly different compared to all other tested conditions ($p < 0.05$, one way ANOVA test).

state levels of cellular oxidants [4,7]. One of the proposed mechanisms for the increase in oxidants is that there is an imbalance between the enzymes that generate and metabolize H_2O_2, resulting in an increase in cellular H_2O_2 concentration [4,34]. On the other hand, the increase in cellular oxidant levels may be secondary to mitochondrial alterations. It has been proposed that zinc deficiency can affect the electron transport chain impairing the complete reduction of O_2 [40]. Possible alterations in the expression of components of the electron transport chain have been recently reported [7]. The rapid increase in H_2O_2 that is observed in the zinc deficient IMR-32 cells suggests that rapid alterations in the mitochondrial respiratory chain or in other early mechanisms could occur, while processes that require new protein synthesis, such as a differential or impaired expression of antioxidant enzymes or components of the respiratory chain, represent late events that contribute to the progression of oxidative stress. Regardless of the mechanism by which the stress is generated, the present results show that the increase in cell oxidant levels and H_2O_2 release associated with zinc deficiency in IMR-32 cells can be prevented when the low zinc cells are simultaneously incubated with antioxidants such as LA or NAC.

In neurons, as in different cell types, glutathione is a fundamental compound in the defense against oxidative stress [41,42]. A homeostatic mechanism for glutathione has been described between astrocytes and neurons, by which astrocytes supply neurons with glutathione precursors [42,43]. Since oxidized glutathione is extremely toxic to neurons, these cells possess specific mechanisms to extrude it out of the cells [42]. In this study, zinc deficiency was associated with low total glutathione concentrations in human neuroblastoma cells. The low concentration of total glutathione could be due to an increase rate of glutathione oxidation, secondary to a rise in intracellular oxidants, with a subsequent extrusion of oxidized glutathione. Supplementation with both LA and NAC restored total glutathione concentrations, presumably due to their capacity to prevent the increase in cellular H_2O_2 and the replenishment of reduced glutathione by NAC.

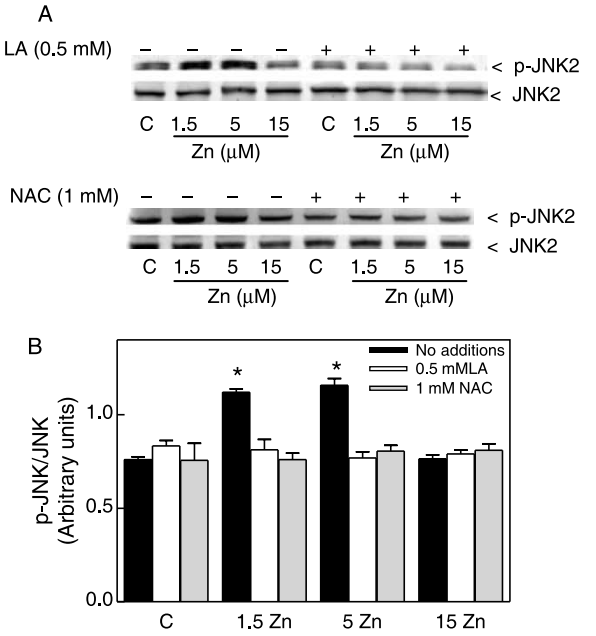

Figure 7. LA and NAC prevented zinc deficiency-induced increase in JNK2 phosphorylation. Total cell fractions were isolated after 24 h of culturing cells in control non-chelated media (C) or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, incubated simultaneously without or with either 0.5 mM LA or 1 mM NAC. (A) Western blots for phosphorylated JNK2 (p-JNK2) and JNK2, after 24 h of incubation under the different treatments. (B)- After quantitation, the ratio p-JNK2 /JNK2 was calculated for cells incubated without (full bars) or with the addition of LA (empty bars) or NAC (grey bars). Results are shown as means ± SEM of 4 independent experiments. *Significantly different compared to all other tested conditions ($p < 0.05$, one way ANOVA test).

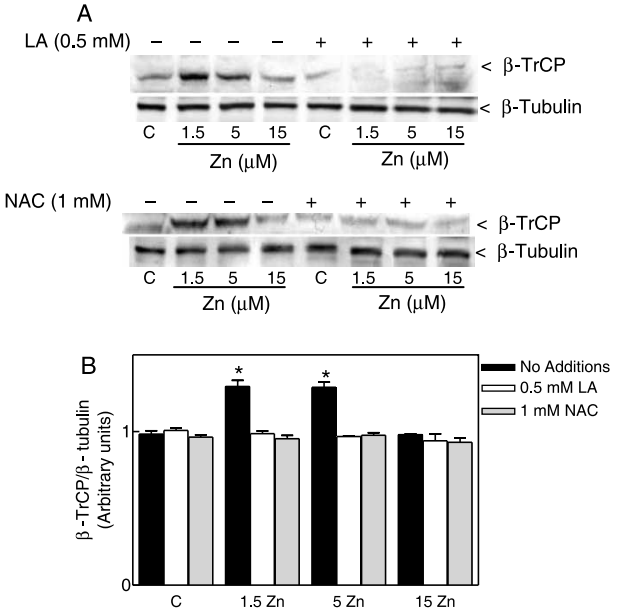

Figure 8. LA and NAC prevented zinc deficiency-induced increase in β-TrCP content. Total cell fractions were isolated after 24 h of culturing cells in control non-chelated media (C) or chelated media containing 1.5 (1.5 Zn), 5 (5 Zn) or 15 (15 Zn) μM zinc, incubated simultaneously without or with either 0.5 mM LA or 1 mM NAC. (A) Western blots for β-TrCP and β-tubulin, after 24 h of incubation under the different treatments. (B) After quantitation, the ratio β-TrCP/β-tubulin was calculated for cells incubated without (full bars) or with the addition of LA (empty bars) or NAC (grey bars). Results are shown as means ± SEM of 3 independent experiments. *Significantly different compared to all other tested conditions ($p < 0.05$, one way ANOVA test).

The NF-κB is a redox-sensitive transcription factor [11–13]. It has been previously shown that an increase in reactive oxygen species [44] or a decrease in intracellular thiols (mainly glutathione) [45] can directly activate NF-κB or induce the phosphorylation of IκBα by activating the IκB kinase complex. In agreement with this, the current study shows that zinc deficiency-induced oxidative stress is associated with an increase in IκBα phosphorylation and a high NF-κB-DNA binding activity in total cell fractions; these effects were prevented by the addition of either LA or NAC. Similarly, in other experimental models, LA and NAC have also been shown to inhibit NF-κB activation [17,46]. It is important to stress that we have previously observed that although the cytosolic events of NF-κB are activated by zinc deficiency, the nuclear transport of the active NF-κB can be impaired secondary to a zinc deficiency-induced alteration in tubulin polymerization [35]. Thus, despite the activation of the cytosolic events of the NF-κB signaling cascade, zinc deficiency can be associated with a decreased transactivation of NF-κB-driven genes [35].

Unlike NF-κB, H_2O_2 and other reactive oxygen species activate AP-1 mainly through the activation of the MAPKs, JNK and p38 [22]. An increase in AP-1-DNA binding activity can be a consequence of zinc deficiency [4]. In zinc deficient IMR-32 cells, an increase in JNK and p38 phosphorylation was observed compared to control cells. The current study shows that LA and NAC can prevent JNK and p38 phosphorylation with a consequent inhibition of AP-1-DNA binding activity. Thus, in zinc deficiency, AP-1 activation can be triggered by an increase of H_2O_2 (or other oxidants) or by alterations in cellular thiols.

JNK can regulate the NF-κB activation cascade by stabilizing β-TrCP's mRNA [39]. β-TrCP mediates the ubiquitination of IκB, through the recruitment of the E3 ubiquitin ligase complex [47]. Thus, β-TrCP activates NF-κB by facilitating the ubiquitination and further proteasome-dependent degradation of the inhibitory peptide IκB. Moreover, β-TrCP participates in the processing of NF-κB2/p100 to generate p52 [48]. The observed increase in p-JNK levels in the zinc deficient cells directed the increase in β-TrCP protein levels, since it was prevented by the simultaneous incubation of cells with either, LA or NAC, indicating a redox regulation for β-TrCP. These results propose a link between JNK regulation and

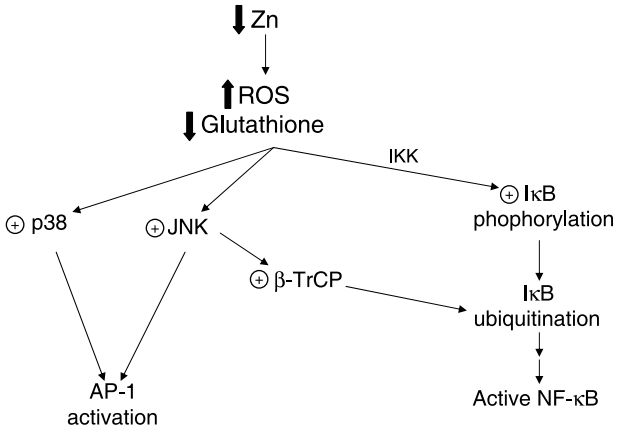

Figure 9. Scheme for the proposed effects of zinc deficiency on AP-1 and NF-κB activation and the interrelationship between the pathways. A decrease in cellular zinc leads to a rapid increase in cellular oxidant levels and to a decrease in total glutathione concentrations. These two events could activate p38 and JNK MAPKs, triggering the activation of transcription factor AP-1. The increase in cellular oxidant levels and the decrease in total glutathione were also associated with the activation of NF-κB through an increase in the phosphorylation of IκBα. A cross-talk between both signalling cascades can occur in zinc deficiency. JNK can contribute to NF-κB activation by stabilizing β-TrCP, a protein that regulates IκBα degradation and p100 processing.

NF-κB activation; suggesting that NF-κB activation due to the increase in oxidants associated with zinc deficiency could be through IKK and IκB phosphorylation pathway and through JNK-β-TrCP-regulated IκB degradation as well as p100 processing.

In summary (Figure 9), results from the current study demonstrate that in neuroblastoma IMR-32 cells a decrease in cellular zinc concentrations triggers the activation of both NF-κB and AP-1 by increasing cellular oxidant levels and/or by decreasing the concentration of cellular glutathione. A cross-talk regulation between both cascades exists at the level of JNK and β-TrCP. LA and NAC may represent new therapeutical approaches to the treatment of zinc-deficiency-associated pathologies.

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