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Zinc deficiency induces production of the proinflammatory cytokines IL-1β and TNFα in promyeloid cells via epigenetic and redox-dependent mechanisms

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Abstract

The deprivation of zinc, caused by malnutrition or as a consequence of aging or disease, strongly affects immune cell functions, causing higher frequency of infections. Among other effects, an increased production of reactive oxygen species (ROS) and proinflammatory cytokines has been observed in zinc-deficient patients, but the underlying mechanisms were unknown. The aim of the current study was to define mechanisms explaining the increase in proinflammatory cytokine production during zinc deficiency, focusing on the role of epigenetic and redox-mediated mechanisms.

Interleukin (IL)-1β and tumor necrosis factor (TNF)α production was increased in HL-60 cells under zinc deficiency. Analyses of the chromatin structure demonstrated that the elevated cytokine production was due to increased accessibilities of IL-1β and TNFα promoters in zinc-deficient cells. Moreover, the level of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase-produced ROS was elevated under zinc deficiency, subsequently leading to p38 mitogen-activated protein kinase (MAPK) phosphorylation. The increased activation of p38 MAPK appeared to be necessary for posttranscriptional processes in IL-1β and TNFα synthesis.

These data demonstrate that IL-1β and TNFα expression under zinc deficiency is regulated via epigenetic and redox-mediated mechanisms. Assuming an important role of zinc in proinflammatory cytokine regulation, this should encourage research in the use of zinc supplementation for treatment of inflammatory diseases.

Keywords: Zinc deficiency; Epigenetics; Gene regulation; Cytokines; Reactive oxygen species

1. Introduction

Zinc is an essential trace element important for a variety of cellular functions such as apoptosis, signal transduction, transcription, differentiation and replication in all organ systems and during embryonic development [1–5]. Therefore, zinc deficiency caused by malnutrition or as a consequence of aging, pregnancy or disease is detrimental for human health [3,5,6] and is currently one of the leading causes of morbidity and mortality in developing countries [7,8].

It has been demonstrated that zinc is necessary for the structure and function of over 300 enzymes [9], including a number of DNA methyltransferases, methyl-binding proteins and histone-modifying enzymes such as acetylases, deacetylases or methylases [10–13]. This along with the observation that zinc deficiency induces global DNA hypermethylation [11,13] points to a role of zinc in epigenetic processes such as chromatin remodeling, DNA methylation, histone modification and noncoding RNA synthesis [10–13].

In addition to its influence on epigenetic processes, zinc also regulates gene expression via its involvement in intracellular signaling [2,14–17]. Zinc is reported to stabilize but also inhibit transcription factors, kinases and phosphatases or the assembly of multiprotein complexes [2,15]. Moreover, the direct regulatory role of zinc in gene expression as a second messenger and its indirect role via modifying calcium flux in cells have been reported [2]. Finally, there are a number of studies describing the role of zinc as an antioxidant as well as the increase of oxidative stress during zinc deficiency [16–19], providing alternative mechanisms for the regulation of gene expression by zinc.

The majority of zinc-regulated genes are involved in signal transduction, in responses to oxidative stress or in growth and energy utilization [3], all known to be particularly important during regulation of the immune response [15]. A variety of studies have already shown a strong impact of zinc deficiency on cell-mediated immunity, including various T-cell defects [1,4,15,20,21]. In contrast, the number and reactivity of myeloid cells increase during zinc deficiency [20]. It has been shown that zinc deficiency induces proinflammatory cytokine synthesis and reactive oxygen production in myeloid cells, but the number of studies is limited and the underlying mechanisms are not completely understood [3,17,22–25].

Therefore, we investigated the influence of zinc deficiency on the production of the proinflammatory cytokines tumor necrosis factor (TNF)α and interleukin (IL)-1β in promyeloid cells, focusing on the role of epigenetic and redox-mediated mechanisms as possible explanations for zinc-deficiency-induced changes.

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2. Methods and materials

2.1. Cell culture

Zinc-sufficient HL-60 cells (HL-60^{suf}) were grown in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% low-endotoxin fetal calf serum (PAA, Coelbe, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all Lonza) in a 5% CO_2 humidified atmosphere at 37°C. For zinc-sufficient THP-1 cells, 0.5% β-mercaptoethanol (Merck, Darmstadt, Germany) was added to the medium. To induce zinc deficiency, cells were cultured with the membrane permeable zinc chelator *N*,*N*′,*N*′-tetrakis-(2-pyridyl-methyl)ethylenediamine (TPEN, 1 μM, Sigma-Aldrich, Taufkirchen, Germany) for 7 days and are denoted as HL-60^{def} and THP-1^{def}. To measure cellular viability, cells were treated as indicated in each experiment and subsequently incubated with propidium iodide (10 μg/ml in phosphate-buffered saline) for 10 min at 4°C in the dark. The dye is membrane impermeable in intact cells and stains dead cells as a result of the loss of plasma membrane integrity. Staining was detected by flow cytometry using a FACSCalibur flow cytometer. For zinc reconstitution (HL-60^{rec}), HL-60^{def} cells were washed and incubated in control medium for another 7 days. Zinc-deficient medium was obtained by treatment with CHELEX 100 ion exchange resin (Sigma-Aldrich), known to bind divalent cations, for 1 h at room temperature, followed by reconstitution of 500 μM $CaCl_2$ and 400 μM $MgCl_2$ [25], being essential for cell proliferation. Other cations need not be reconstituted as shown by Yui et al. [26]. HL-60 cells cultured in chelexed medium are denoted HL-60^{CHE}. As a zinc-sufficient control, chelexed medium was supplemented with 8 μM $ZnSO_4$ (HL-60^{CHE+}). The differentiation of HL-60 cells into monocytic cells using 1α,25-dihydroxyvitamin D_3 (VD3; 100 nM) for 72 h was performed and monitored as described [25,27].

2.2. Enzyme-linked immunosorbent assay (ELISA)

Supernatants were harvested and stored at −20°C, and IL-1β and TNFα were quantified by ELISA (BD Pharmingen).

2.3. Reverse transcription and real-time polymerase chain reaction (PCR)

RNA was isolated using RNA II-Kit (Macherey-Nagel, Düren, Germany) and reverse-transcribed using qScript cDNA Synthesis Kit in reactions containing 50 ng/μl RNA (Quanta Bioscience, Gaithersburg, MD, USA). Primers for IL-1β [28], TNFα (forward primer: 5′-ATGAGCACTGAAAGCATGATCC-3′; reverse primer: 5′-GAGGGCT-GATTAGAGAGAGGTC-3′) and the housekeeping gene *Porphobilinogen-deaminase* (PBGD) [29] were added at final concentrations of 0.1 μM. To exclude amplification of genomic DNA or hnRNA, primer pairs which exclusively bind to exon–exon borders or within different exons were chosen. IL-1β and TNFα real-time PCRs were performed with 2 μl cDNA in 25-μl reaction volumes in duplicates using Brilliant Sybr Green qPCR Master Mix (Applied Biosystems, Darmstadt, Germany) with the following parameters: 95°C for 15 min followed by 40 cycles of 95°C for 30 s and 56°C for 30 s. Standard curves were generated using 10-fold serial dilutions of cDNA from peripheral blood mononuclear cells. The mRNA levels of the cytokines were normalized to PBGD levels.

2.4. Chromatin accessibility by real-time (CHART)-PCR assay

MNase accessibility assays were performed, and results were plotted as described previously [27]. Real-time PCR was performed in 25-μl reaction volumes in duplicates using Brilliant Sybr Green qPCR Master Mix (Applied Biosystems) containing 100 ng of DNA. Primers for IL-1β promoter regions IL-1β I, II, IV and VIII [30]; TNFα promoter regions TNF I–IV [31]; or the GAPDH promoter [32] were added at a final concentration of 0.1 μM. For quantification, a standard curve was generated using serial dilutions of genomic DNA. MNase accessibility was calculated by the following formula: $a = \left|100-\left(\frac{\text{quantity(MNase+)}}{\text{quantity(MNase−)}}\cdot 100\right)\right|\%$

2.5. Measurement of free intracellular zinc with FluoZin-3AM

Free zinc was measured as described previously [33] using FluoZin-3 AM ester (1 μM, Invitrogen, Karlsruhe, Germany). The zinc-dependent fluorescence was analyzed with FACScan (BD Bioscience) using Cellquest software 3.0. The concentration of intracellular labile zinc was calculated from the mean fluorescence with the formula $[Zn]=K_D\times[(F-F_{min})/(F_{max}-F)]$ using a dissociation constant for the Zn/FluoZin-3AM complex of 8.9 nM [34] and determining the maximal and minimal fluorescence by addition of zinc (100 μM) and pyrithione (50 μM) or TPEN (50 μM), respectively.

2.6. Measurement of reactive oxygen species (ROS) production using dihydrorhodamine 123 (DHR)

A total of 1×10^6 cells/ml were loaded with DHR (1 μg/ml, Invitrogen) in incubation buffer (5 mM glucose, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 120 mM NaCl, 25 mM Hepes, 5.4 mM KCl, 0.3% bovine serum albumin; pH 7.35) for 30 min at 37°C. Subsequently, cells were washed with measurement buffer (incubation buffer without albumin) and transferred into a 96-well plate at a density of 1×10^6 cells/ml. The resulting fluorescence was recorded on a fluorescence well plate reader (excitation wavelength: 485 nm, emission: 535 nm, Ultra 384, Tecan, Crailsheim, Germany).

2.7. Cell extracts and Western blotting

A total of 2×10^6 cells were lysed and sonicated in 100 μl lysis buffer [0.5 M Tris–HCl (pH 6.8), 26.6% glycerin, 10% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4 and 1% β-mercaptoethanol] [27]. SDS–polyacrylamide gel electrophoresis using an equivalent of 4×10^5 cells and Western Blot analysis were performed as described previously [27]. Membranes were incubated with horseradish-peroxidase (HRP)-linked anti-rabbit IgG secondary antibody and HRP-coupled anti-biotin antibody for detection of biotin-labeled molecular weight standard for 1 h, followed by detection with LumiGlo reagent (Cell Signaling Technology) on a LAS-3000 (Fujifilm Lifescience, Düsseldorf, Germany). The membrane was stripped, blocked and then reprobed for β-actin as described [27].

2.8. Statistical analysis

Statistical significance of experimental results was analyzed by Student's *t* test or, in case of multiple comparisons, by one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett´s honestly significant difference post hoc test using GraphPad Prism software version 5 (GraphPad software, La Jolla, CA, USA). For single comparisons, $^*P<.05$ and $^*P<.01$ are used for data significantly different from the respective HL-60^{suf} as determined by ANOVA/Dunnett's honestly significant difference test or Student's *t* test. For multiple comparisons, significant differences at $P<.05$, determined by ANOVA/Tukey's honestly significant difference test, are indicated by different letters.

3. Results

3.1. Impact of zinc deficiency on intracellular free zinc as well as IL-1β and TNFα expression

Zinc deficiency induces proinflammatory cytokine expression in myeloid cells [22,27], but the underlying mechanisms are unknown. To examine the effect of zinc deficiency on IL-1β and TNFα expression, HL-60 cells, producing negligible proinflammatory IL-1β and TNFα amounts [27], were incubated with TPEN.

First, we verified that long-term depletion of zinc by TPEN significantly decreased free intracellular zinc levels in HL-60 cells compared to HL-60^{suf} (Fig. 1A), while not affecting viability of the cells (Supplemental figure 1). Moreover, unstimulated HL-60^{def} produced small amounts of IL-1β and TNFα mRNA which were significantly increased after stimulation with phorbol-12-myristate-13-acetate (PMA) only (Fig. 1B, C). Low expression of IL-1β and TNFα mRNA was also detected in unstimulated HL-60^{suf}, but no significant increase was observed after stimulation with PMA (Fig. 1B, C). Lipopolysaccharide (LPS) generally had no effect, indicating that HL-60^{def} cells were not differentiated and CD14 negative [25]. This suggests a positive regulatory role of long-term zinc deficiency in PMA-induced IL-1β and TNFα transcription.

To check whether zinc deficiency also induced the secretion of IL-1β and TNFα by HL-60 cells, we analyzed their quantities in the supernatants of the cells by ELISA. The basal amount of IL-1β released by HL-60^{def} was higher than by HL-60^{suf} (Fig. 2A), but did not reach significance. IL-1β protein levels further increased after PMA stimulation in HL-60^{def} only (Fig. 2A). PMA treatment increased TNFα secretion by HL-60^{def} and HL-60^{suf} (Fig. 2B). However, the amounts of TNFα detected in the supernatants of HL-60^{def} were significantly higher than those of PMA-stimulated HL-60^{suf}, reflecting the mRNA data. Reconstitution of HL-60^{def} cells with control medium for another 7 days (Fig. 2A, B) showed the reversibility of the changes induced by zinc deficiency. The low levels of IL-1β and TNFα protein in the supernatants after zinc reconstitution were comparable to those detected for HL-60^{suf}.

3.2. Chromatin remodeling within IL-1β and TNFα promoters

Recent results showed that chromatin remodeling within IL-1β and TNFα promoters into an open structure is important for the activation of IL-1β and TNFα expression [27,31]. Because zinc is involved in epigenetic processes such as chromatin remodeling [11–13,35], we compared the chromatin structures of IL-1β and TNFα promoters in HL-60^{def} and HL-60^{suf}. Accessibilities of promoter regions IL-1β I (−107 to −17), IL-1β II (−199 to −109) (Fig. 3A),

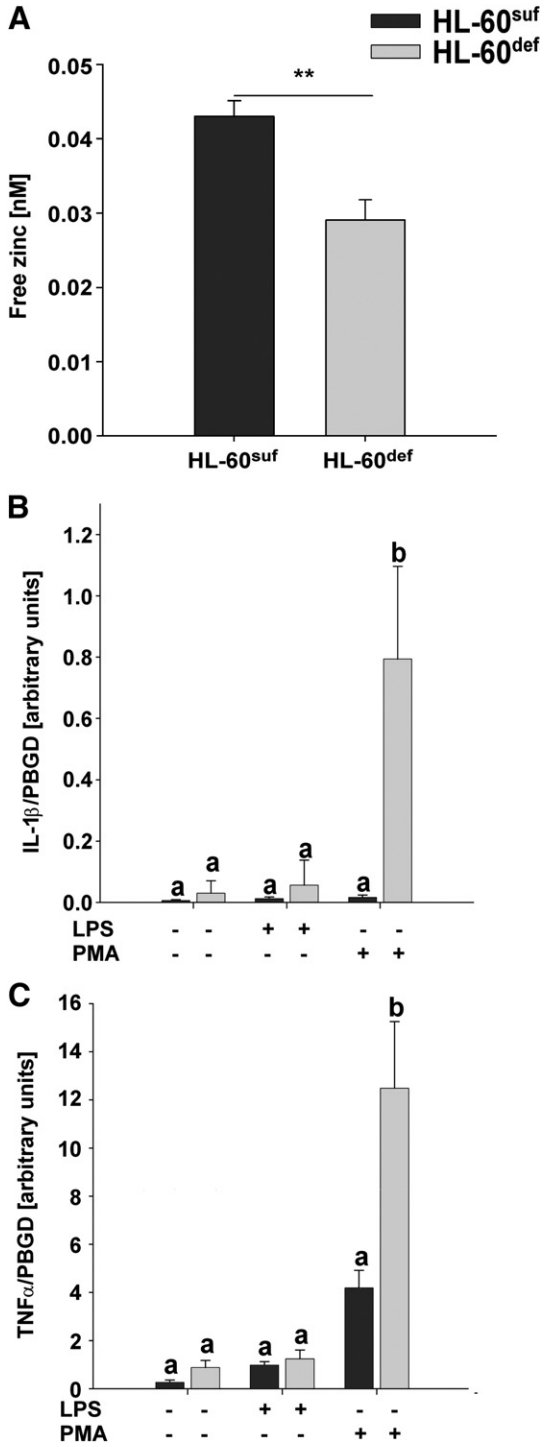


Fig. 1. Impact of intracellular free zinc levels on IL-1β and TNFα mRNA expression. (A) HL-60^{suf} (black) and HL-60^{def} (light grey) cells were loaded with the zinc-specific fluorescent probe FluoZin-3, and zinc-dependent fluorescence was recorded by flow cytometry. Results represent means±S.E. (S.E.M.) of $n=7$ independent experiments. Significant differences at $^{**}P<.01$ were determined by Student's t test. (B-C) HL-60^{suf} (black) and HL-60^{def} (light grey) cells were stimulated with LPS (250 ng/ml) or PMA (10 ng/ml) for 3 h. IL-1β (B) and TNFα (C) mRNA was analyzed by quantitative real-time PCR. Values were normalized to housekeeping gene PBGD and are presented as means± S.E. of $n=8$ independent experiments. Significant differences at $P<.001$, determined by ANOVA/Tukey's honestly significant difference test, do not share the same letters.

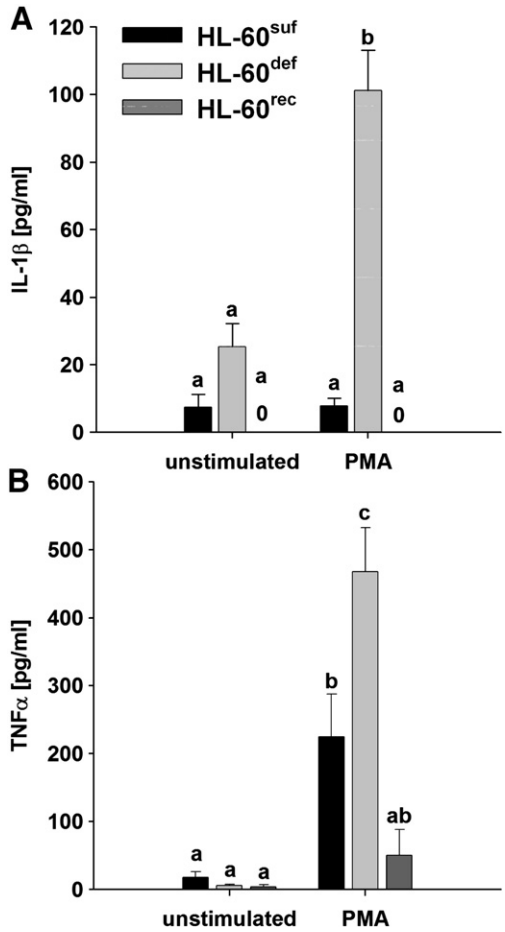


Fig. 2. Zinc deficiency and proinflammatory IL-1β and TNFα release. HL-60^{suf} (black), HL-60^{def} (light grey) and HL-60^{rec} (dark grey) cells were cultured as described in Materials and methods. After stimulation with PMA (10 ng/ml) for 3 h, the amounts of (A) IL-1β and (B) TNFα in the culture supernatants were measured by ELISA. Results shown are means±S.E.M. of $n=6$ independent experiments. 0 indicates that no cytokine secretion could be detected. Significant differences at $P<.05$, determined by ANOVA/Tukey's honestly significant difference test, do not share the same letters.

TNFα I (+99/–42) and TNFα II (+32/–119) (Fig. 3B) were significantly increased under zinc deficiency, demonstrating that IL-1β and TNFα promoters become highly accessible under zinc deficiency near the transcriptional start sites. Zinc reconstitution decreased the accessibilities of promoter regions IL-1β I, IL-1β II, TNFα I and TNFα II compared to the structures observed in HL-60^{def}, resembling the inaccessible structure detected in HL-60^{suf}.

The accessibility of promoter region TNFα III (−100/−250) rose from 43% to 59% under zinc deficiency (Fig. 3B), without reaching significance. Analyses of regions IL-1β IV (−347 to −257), IL-1β VIII (−673 to −583) and TNF IV (−195/–345), which are located further upstream, revealed their complete inaccessibility under all conditions (data not shown). Additionally, zinc deficiency had no effect on human GAPDH promoter accessibility (data not shown), demonstrating the specific influence of zinc on these proinflammatory gene promoters.

3.3. Specificity of the zinc-deficiency-induced changes

To exclude TPEN specific side effects other than intracellular zinc chelation, we analyzed HL-60 cells cultured in zinc-deficient, chelexed medium (HL-60^{CHE}) in comparison to HL-60^{CHE+} supplemented with 8 μM $ZnSO_4$ (Supplemental Figure 2). Moreover, to rule out a cell-specific phenomenon, the monocytic cell line THP-1 was used. Significantly decreased intracellular zinc content in TPEN-cultured THP-1^{def} (Supplemental Figure 3A) and in HL-60^{CHE} (Supplemental Figure 2A) as well as an increase in IL-1β and TNFα secretion (Supplemental Figures 2B–C and 3B–C) in these cells could be

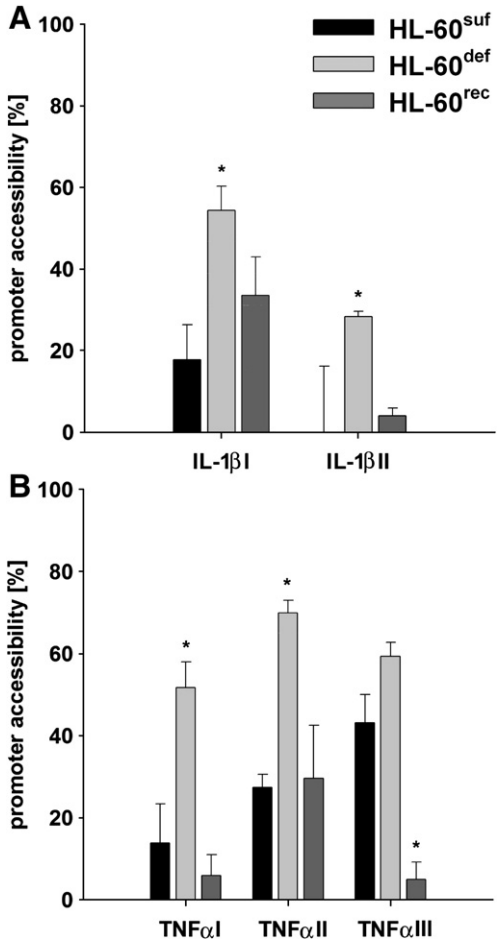


Fig. 3. Impact of zinc deficiency on IL-1β and TNFα promoter conformation. CHART-PCR analyses of IL-1β and TNFα promoters in HL-60 cells are shown. HL-60^{suf} (black), HL-60^{def} (light grey) and HL-60^{rec} (dark grey bars) cells were cultured as described in Materials and methods. Real-time PCR was performed using primer sets for (A) IL-1β promoter regions I and II and for (B) TNFα promoter regions I, II and III. Mean calculated accessibilities and S.E.M. for $n=4$ independent experiments are presented. *$P<.05$ for data significantly different from respective HL-60^{suf} were determined by ANOVA/Dunnett's honestly significant difference test.

demonstrated. Additionally, promoter accessibilities of IL-1β I, IL-1β II, TNFα I and TNFα II were higher in THP-1^{def} and HL-60^{CHE} than in THP-1^{suf} or HL-60^{CHE+}, respectively (Supplemental Figures 2D–E and 3D–E).

3.4. Role of zinc deficiency in HL-60 differentiation into monocytic cells

Since VD3-induced differentiation of HL-60 cells leads to IL-1β promoter remodeling and IL-1β and TNFα production, and combined zinc deficiency enhances CD11b/CD14 surface expression [27,38], CD14 expression was investigated under zinc-deficient conditions. No expression of the monocyte marker CD14 on stimulated and unstimulated HL-60^{def} or HL-60^{CHE} (Supplemental Figure 4A–D) or after TPEN incubation of HL-60 cells for 14 days could be detected. In contrast, VD3 incubated cells showed 58% CD14^{+} cells (Supplemental Figure 4E–F). This suggests that the remodeling of IL-1β and TNFα promoters induced by zinc deficiency was a separate effect independent from complete differentiation.

3.5. ROS production of zinc-deficient HL-60 cells

Zinc deficiency is described to elevate oxidative stress in different cell types [18,19]. We observed that the basal level of ROS was significantly higher in HL-60^{def} than in HL-60^{suf} (Fig. 4A), indicating a shift to a more intracellular oxidative milieu under zinc deficiency.

Whereas no changes in DHR oxidation after LPS or PMA stimulation of HL-60^{suf} (Fig. 4B) could be detected, ROS concentrations in HL-60^{def} steadily increased only after PMA stimulation until the end of the experiment (Fig. 4C). In HL-60^{rec}, only a small increase of DHR oxidation could be detected shortly after PMA stimulation (Fig. 4D), indicating the reversibility of the changes in PMA-induced ROS synthesis that we observed in HL-60^{def}. LPS had no influence on ROS production in HL-60^{rec} as observed in HL-60^{suf} and HL-60^{def} (Fig. 4D).

In myeloid cells, ROS are primarily produced by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase (NOX) during oxidative burst [36]. Preincubation of HL-60^{suf}, HL-60^{def} and HL-60^{rec} with the NOX inhibitor diphenyleneiodonium (DPI) before PMA stimulation did not alter the basal levels of ROS production in these cells (Fig. 4E–G). In contrast, DPI preincubation almost completely abrogated the PMA-induced increase in DHR oxidation in HL-60^{def} (Fig. 4F). This indicates that ROS are produced by NOX in HL-60^{def}.

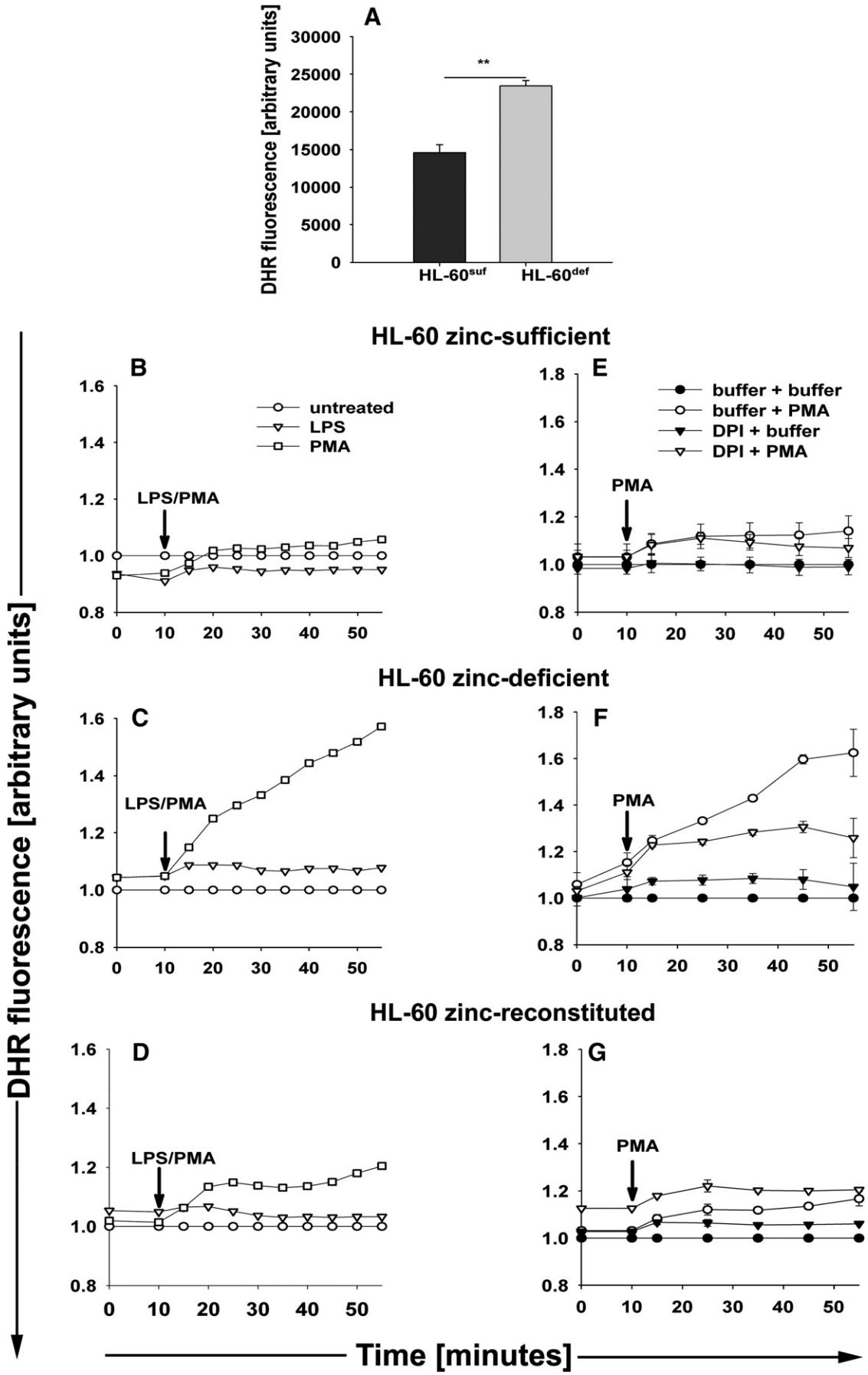
3.6. Connection between zinc, ROS and cytokine expression

To elucidate whether changes in the ROS production are related to cytokine expression under zinc deficiency, cells were again preincubated with DPI before stimulation, and IL-1β and TNFα release was measured by ELISA. DPI abrogated the PMA-induced increase of IL-1β secretion in HL-60^{def} (Fig. 5A), whereas the basal IL-1β expression remained unchanged. In addition, we found a significant decrease in PMA-induced TNFα synthesis in HL-60^{suf} and HL-60^{def} preincubated with DPI (Fig. 5B), suggesting that ROS-dependent and ROS-independent pathways are involved in PMA-induced TNFα synthesis.

To more precisely define how ROS influence IL-1β and TNFα expression under zinc deficiency, we assessed IL-1β and TNFα promoter accessibilities after treatment with *N*-acetylcysteine (NAC) for 7 days. The strong antioxidant NAC was able to abrogate PMA-induced DHR 123 oxidation in HL-60^{def} (Supplemental Figure 5). As depicted in Fig. 6A–B, coincubation of HL-60^{def} with NAC did not inhibit the remodeling of promoter regions IL-1β I, IL-1β II, TNFα I and TNFα II observed in HL-60^{def}, but further increased the promoter accessibilities of these regions. Control regions IL-1β IV, IL-1β VIII and TNFα IV remained inaccessible (data not shown). Hence, these results excluded that ROS are involved in chromatin remodeling of IL-1β and TNFα promoters.

Next, we investigated whether ROS induced transcription of IL-1β and TNFα. Fig. 7 shows that NAC had no influence on IL-1β and TNFα mRNA levels in HL-60^{def}. Additionally, preincubation of HL-60^{def} with DPI had also no significant effect on PMA-induced IL-1β or TNFα mRNA expression (Supplemental Figure 6). Hence, it was excluded that the ROS-dependent increase of IL-1β and TNFα secretion was regulated via chromatin remodeling (Fig. 6) or changes in transcription (Fig. 7).

Fig. 4. Changes in the redox status during zinc deficiency. HL-60^{suf}, HL-60^{def} and HL-60^{rec} cells were cultured as described in Materials and methods and loaded with DHR123 for 30 min. The fluorescence resulting from DHR123 oxidation was measured in a well plate reader. (A) Basal levels of oxidated DHR123 fluorescence are shown as mean ±S.E.M. of $n=7$ independent experiments. Significant differences from HL-60^{suf} at **$P<.01$ were determined by Student's *t* test. (B–D) After 10 min of recording of the baseline fluorescence for HL-60^{suf} (B), HL-60^{def} (C) and HL-60^{rec} (D), the cells were stimulated with buffer (circles), LPS (250 ng/ml, triangles) or PMA (10 ng/ml, squares) for another 45 min, and fluorescence was monitored and normalized to untreated controls. One representative example of $n=3$ for each approach is shown. (E–G) DHR123 loaded HL-60^{suf} (E), HL-60^{def} (F) and HL-60^{rec} (G) cells were preincubated with buffer (circles) or the NOX inhibitor DPI (10 μM, triangles) for 30 min. After 10 min of recording of the baseline, buffer (filled symbols) or PMA (10 ng/ml, open symbols) was added, and fluorescence was monitored for another 45 min and normalized to buffer-treated controls. Shown are mean ±S.E.M. of at least $n=3$ independent experiments for each type of cell culture.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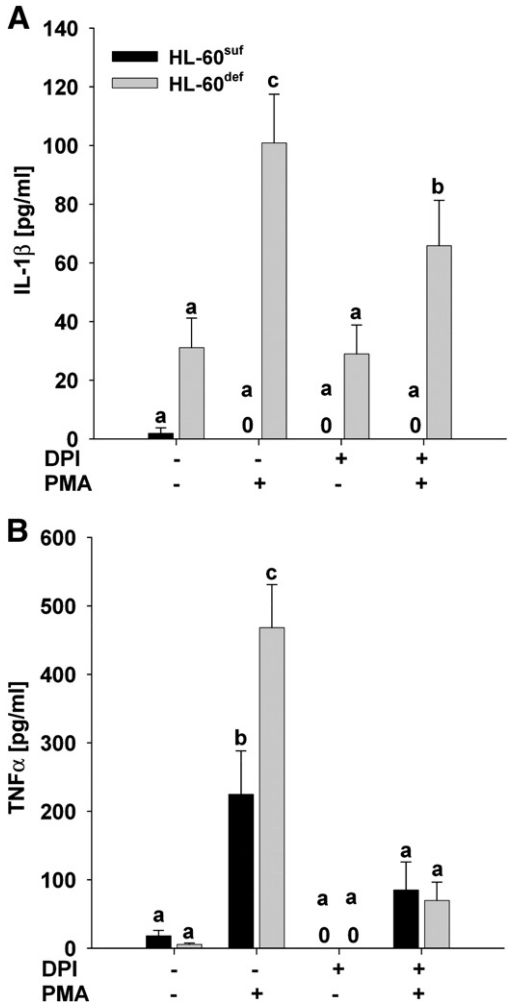

Fig. 5. Role of ROS in IL-1β and TNFα synthesis. After preincubation with the NOX inhibitor DPI (10 μM) for 30 min, HL-60^{suf} (black) and HL-60^{def} (light grey) cells were stimulated with PMA (10 ng/ml) for 3 h as indicated. IL-1β (A) and TNFα (B) secretion was measured by ELISA. Data are presented as mean ±S.E.M. for $n=13$ independent experiments. 0 indicates that no cytokine could be detected. Significant differences at $P<.05$, determined by ANOVA/Tukey's honestly significant difference test, do not share the same letters.

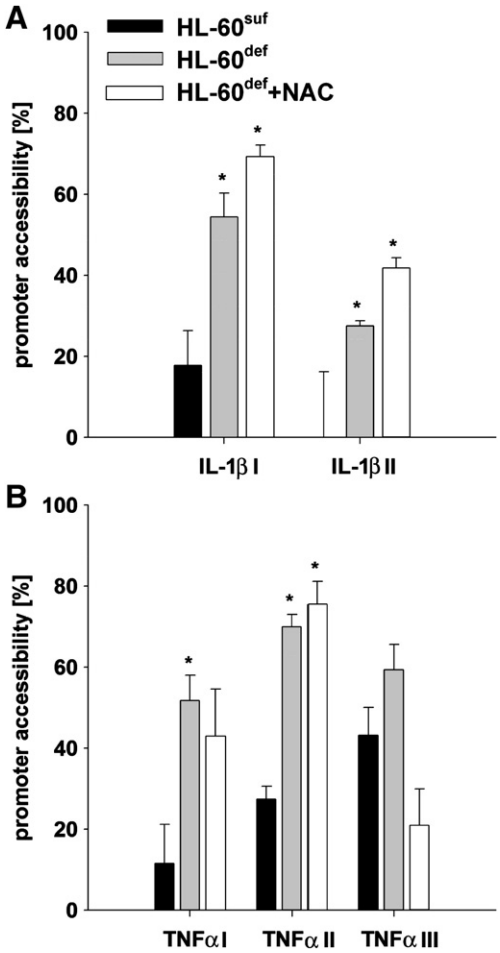

Fig. 6. Role of antioxidants in chromatin remodeling. HL-60 cells were incubated with (light grey bars) or without (black bars) TPEN or TPEN in the presence of the antioxidant NAC (1 mM, white bars) for 7 days followed by CHART-PCR analysis. CHART assay was performed using primer sets for IL-1β promoter regions I and II (A) as well as for TNFα promoter regions I, II and III (B). Mean calculated accessibilities ±S.E.M. for $n=4$ independent experiments are shown. *$P<.05$ for data significantly different from the respective HL-60^{suf} was determined by ANOVA/Dunnett's honestly significant difference test.

3.7. Influence of zinc and ROS on p38 MAPK signaling

Since p38 mitogen-activated protein kinase (MAPK) is involved in IL-1β and TNFα production in monocytes [24,30] and in gene expression linked to ROS [37], the role of p38 and ROS in the posttranscriptional processing of IL-1β and TNFα was analyzed. PMA stimulation led to a fast increase in p38 MAPK activation within 15 min only in HL-60^{def} but not in HL-60^{suf} (Fig. 8). The detection of p38 phosphorylation in both HL-60^{def} and HL-60^{suf} after H_2O_2 treatment verified a ROS-mediated activation of p38 MAPK (Supplemental Figure 7A). Moreover, DPI preincubation blocked PMA-induced phosphorylation of p38, underlining the role of NOX-produced ROS in p38 MAPK activation (Supplemental Figure 7B).

Finally, we investigated whether p38 MAPK activation by ROS was essential for posttranscriptional processing of IL-1β and TNFα. Preincubation with the p38 inhibitor SB202190 had no effect on IL-1β or TNFα secretion in HL-60^{suf} (Fig. 9) but significantly inhibited PMA-induced IL-1β (Fig. 9A) and TNFα secretion (Fig. 9B) in HL-60^{def}. These data demonstrate the important role of ROS-activated p38 in the posttranscriptional processing of IL-1β and TNFα.

4. Discussion

Recent studies suggest a strong influence of zinc in the regulation of proinflammatory cytokine expression in mononuclear cells [15,22,24]. Furthermore, increased IL-1β and TNFα cytokine synthesis during zinc deficiency induced by dietary means, as a consequence of leishmaniasis or cancer, or due to aging or pregnancy was reported [5,22,38]. TPEN-mediated decrease of intracellular zinc levels in promyeloid HL-60 cells (Fig. 1A) is linked to increased IL-1β and TNFα mRNA and protein levels (Figs. 1B–C and 2), supporting the hypothesis that zinc deficiency generates a proinflammatory environment in the human body [5,20,21,39].

During VD3-induced differentiation of promyeloid HL-60 cells, intracellular free zinc levels decrease, whereas IL-1β and TNFα expression increases and the IL-1β promoter is remodeled into an open conformation [25,27]. Interestingly, TPEN treatment can also support 72-h VD3-induced differentiation. Our experiments indicate that the decrease of intracellular zinc due to long-term zinc deprivation promotes changes of the chromatin structures of IL-1β and TNFα promoters enabling the expression of both genes (Figs. 1–3). These observations could be confirmed using zinc-deficient medium, generated using CHELEX 100 (Supplemental Figure 2). To

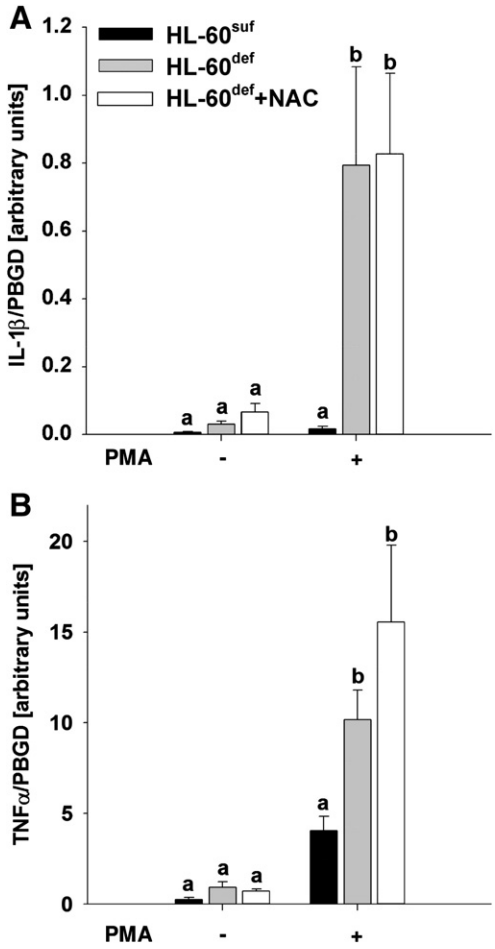

Fig. 7. Effect of ROS on cytokine transcription. To measure the effect of zinc-deficiency-induced ROS on IL-1β and TNFα mRNA expression, cells were incubated with normal medium (black bars), TPEN (1 μM, light grey bars) or a combination of TPEN (1 μM) and NAC (2 mM) (white bars) for 7 days. Cells were stimulated with PMA (10 ng/ml) for 3 h as indicated, and IL-1β and TNFα mRNA expression was measured by real-time PCR. Data are presented as mean ±S.E.M. of $n=8$ independent experiments. Significant differences at $P<.05$, determined by ANOVA/Tukey's honestly significant difference test, are indicated by different letters.

exclude a single cell-specific phenomenon, THP-1 could also confirm the data generated with HL-60 cells (Supplemental Figure 3). Since CD14 is not induced on HL-60^{def} cell surface (Supplemental Figure 4), zinc deficiency alone does not initiate complete monocytic differentiation, but might be able to support VD3-induced differentiation by the activation of some monocytic genes.

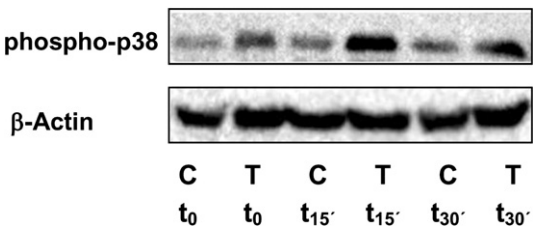

Fig. 8. Influence of PMA on p38 MAPK activity. The effect of zinc chelation by TPEN on p38 MAPK phosphorylation was investigated in HL-60^{suf} (C) and HL-60^{def} (T) stimulated with PMA (10 ng/ml) for 0, 15 and 30 min. p38 phosphorylation was analyzed by Western blotting with antibodies against phosphorylated p38 MAPK (Thr^{180}/Tyr^{182}), p38 MAPK and β-actin. The blot shown is representative of $n=6$ independent experiments.

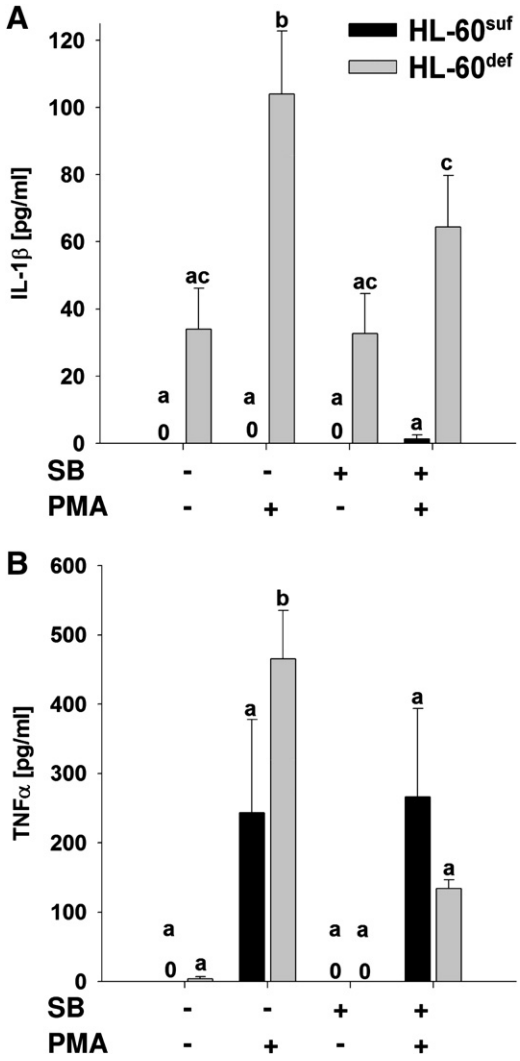

Fig. 9. Role of p38 MAPK in posttranscriptional processing of IL-1β and TNFα. HL-60^{suf} (black bars) and zinc-deficient (grey bars) HL-60 cells were stimulated with PMA (10 ng/ml) for 3 h after preincubation with the p38 MAPK inhibitor SB202190 (SB, 2 μM, 30 min), as indicated. The concentration of IL-1β and TNFα in the supernatants of the cells was determined by ELISA. Data are presented as mean ±S.E.M. of $n=13$ independent experiments. 0 indicates that no cytokine could be detected. Significant differences at $P<.05$, determined by ANOVA/Tukey's honestly significant difference test, do not share the same letters.

The mechanism of how zinc deficiency is involved in chromatin remodeling is currently unclear. DNA demethylation and histone methylation, both known to be affected by zinc [11,13], as mechanisms for chromatin remodeling in the IL-1β promoter could be excluded because inhibition of DNA methyltransferases via 5-aza-2-deoxy-3-cytidine did not result in an increased IL-1β promoter accessibility (data not shown). However, there is a complex network of zinc-regulated molecules and signaling pathways [5,21,39] that may be involved in zinc-deficiency-induced chromatin remodeling.

Since free radicals formation depends on the cellular zinc concentrations [16–19], the alteration of the redox state of the cells could be another important mechanism in the regulation and expression of IL-1β and TNFα. Long-term zinc deprivation of HL-60 cells led to a rise in basal ROS levels compared to HL-60^{suf} (Fig. 4A). We could not detect an LPS-induced increase in ROS production in HL-60^{def}, but observed a strong elevation of ROS after PMA stimulation (Fig. 4B) since HL-60 cells lack CD14 expression.

In primary monocytes, PMA stimulation activates NOX-mediated ROS production [35,40]. NOX is also involved in the PMA-induced

production of ROS by HL-60^{def}, and NOX-produced ROS are important for IL-1β and TNFα production (Fig. 4C). Interestingly, the amount of TNFα in the supernatants of DPI-preincubated, PMA-stimulated HL-60^{def} and HL-60^{suf} was still clearly higher than that in unstimulated cells. These data suggest an additional TNFα-inducing pathway activated by PMA, independent from NOX-produced ROS. Protein kinase C shows broad substrate specificity and functions as a central signal amplifier by phosphorylating different signaling molecules [40], providing a variety of candidate mediators for PMA-induced TNFα expression. Other mechanisms are the deactivation of A20, which is able to block nuclear factor (NF)-κB activation if highly expressed, or a decrease in phosphodiesterase-1 activation leading to protein-kinase-A-induced inhibition of NF-κB activation [17,23]. Additionally, an increase in mRNA stability of both cytokines by a zinc-deficiency-induced decrease in tristetraprolin is discussed [41].

Oxidative stress is known to influence chromatin remodeling, transcription and translation [36,42]. However, we did not observe any decrease in chromatin remodeling of IL-1β and TNFα promoters or in IL-1β and TNFα mRNA levels when ROS were neutralized via NAC or when the production of ROS was blocked via DPI in HL-60^{def} (Figs. 6 and 7). Therefore, we excluded an effect of ROS on chromatin structure and transcription of IL-1β and TNFα genes.

Next, we investigated the role of p38, a redox-sensitive kinase [37] known to be involved in IL-1β and TNFα production [24,29]. An abrogation of *Escherichia-coli*-induced p38 phosphorylation was described when leukocytes were loaded with TPEN 30 min prior to stimulation [24]. In contrast, Zago et al. showed the induction of p38 phosphorylation in zinc-deficient neuronal IMR-32 cells [43], which is in concordance with our rapidly activated p38 in PMA-stimulated HL-60 incubated with TPEN for 7 days (Fig. 8). This indicates a time-dependent effect of zinc deficiency on p38 activation. Because the same effect was detected in H_2O_2-stimulated HL-60 cells but blocked by DPI preincubation, p38 activation seems to be mediated by ROS via PMA-activated NOX.

Results concerning the function of p38 in TNFα and IL-1β synthesis are contradictory. There are studies reporting that p38 is involved in transcription via NFκB or AP-1 [43–45], whereas others claim an influence on mRNA stability or even the regulation of (post) translational events by p38 [24,37]. Inhibition of p38 activation by SB202190 did not abrogate PMA-induced IL-1β or TNFα mRNA production in HL-60^{def} (data not shown), but significantly decreased the secretion of both cytokines into the supernatant (Fig. 9). Therefore, our study supports the posttranscriptional involvement of p38 in IL-1β and TNFα production as suggested by others [24,37].

In conclusion, our results reveal that zinc deficiency leads to chromatin remodeling, facilitating IL-1β and TNFα mRNA transcription after appropriate stimulation. Moreover, zinc deficiency enables PMA-induced ROS production by NOX, subsequently promoting posttranscriptional processing and secretion of IL-1β and TNFα via p38 MAP kinase. Our findings provide a link between zinc deficiency and the induction of IL-1β and TNFα production via epigenetic as well as oxidant-mediated signaling pathways.

Transient hypozincemia is a physiological effect due to hepatic zinc uptake during systemic inflammation induced by proinflammatory cytokines [14,46]. We hypothesize that a prolonged decrease of serum zinc causes increased IL-1β and TNFα production, initialing a vicious circle and inducing permanent zinc uptake by the liver. Subsequently, this would explain the steadily increased elevated level of proinflammatory cytokines as well as the development of chronic inflammation. It is also responsible for tissue destruction, fetal defects and immune dysfunctions reported during zinc deprivation [1,4–6,20].

The reversibility of IL-1β and TNFα expression and chromatin remodeling of their promoters together with the positive results of zinc supplementation studies [5,7,21,47] is a promising hint for the use of zinc in the treatment of chronic inflammatory diseases. The mechanisms that induce IL-1β and TNFα synthesis under zinc deficiency may affect malnutrition, pregnancy and aging, often accompanied by zinc deficiency; thus, epigenetic effects should be investigated in future zinc supplementation trial, e.g., in elderly.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.06.007.

References

[1] Wellinghausen N, Rink L. The significance of zinc for leukocyte biology. J Leukoc Biol 1998;64:571–7.
[2] Beyersmann D, Haase H. Functions of zinc in signaling, proliferation and differentiation of mammalian cells. Biometals 2001;14:331–41.
[3] Cousins RJ, Blanchard RK, Moore JB, Cui L, Green CL, Liuzzi JP, et al. Regulation of zinc metabolism and genomic outcomes. J Nutr 2003;133:1521S–6S.
[4] Honscheid A, Rink L, Haase H. T-lymphocytes: a target for stimulatory and inhibitory effects of zinc ions. Endocr Metab Immune Disord Drug Targets 2009;9:132–44.
[5] Black RE. Micronutrients in pregnancy. Br J Nutr 2001;85(Suppl. 2):S193–7.
[6] Prasad AS, Beck FW, Bao B, Fitzgerald JT, Snell DC, Steinberg JD, et al. Zinc supplementation decreases incidence of infections in the elderly: effect of zinc on generation of cytokines and oxidative stress. Am J Clin Nutr 2007;85:837–44.
[7] Yakoob MY, Theodoratou E, Jabeen A, Imdad A, Eisele TP, Ferguson J, et al. Preventive zinc supplementation in developing countries: impact on mortality and morbidity due to diarrhea, pneumonia and malaria. BMC Public Health 2011;11(Suppl. 3):S23.
[8] Guilbert JJ. The world health report 2. Educ Health (Abingdon) 2003;16:230.
[9] Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. Physiol Rev 1993;73:79–118.
[10] Coneyworth LJ, Mathers JC, Ford D. Does promoter methylation of the SLC30A5 (ZnT5) zinc transporter gene contribute to the ageing-related decline in zinc status? Proc Nutr Soc 2009;68:142–7.
[11] Davis CD, Uthus EO. DNA methylation, cancer susceptibility, and nutrient interactions. Exp Biol Med (Maywood) 2004;229:988–95.
[12] Lu Q, Yang YT, Chen CS, Davis M, Byrd JC, Etherton MR, et al. Zn2+−chelating motif-tethered short-chain fatty acids as a novel class of histone deacetylase inhibitors. J Med Chem 2004;47:467–74.
[13] Wallwork JC, Duerre JA. Effect of zinc deficiency on methionine metabolism, methylation reactions and protein synthesis in isolated perfused rat liver. J Nutr 1985;115:252–62.
[14] Cousins RJ, Liuzzi JP, Lichten LA. Mammalian zinc transport, trafficking, and signals. J Biol Chem 2006;281:24085–9.
[15] Haase H, Rink L. Functional significance of zinc-related signaling pathways in immune cells. Annu Rev Nutr 2009;29:133–52.
[16] Mackenzie GG, Zago MP, Erlejman AG, Aimo L, Keen CL, Oteiza PI. alpha-Lipoic acid and *N*-acetyl cysteine prevent zinc deficiency-induced activation of NF-kappaB and AP-1 transcription factors in human neuroblastoma IMR-32 cells. Free Radic Res 2006;40:75–84.
[17] Prasad AS, Bao B, Beck FW, Sarkar FH. Zinc-suppressed inflammatory cytokines by induction of A20-mediated inhibition of nuclear factor-kappaB. Nutrition 2010.
[18] Maret W. Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxid Redox Signal 2006;8:1419–41.
[19] Prasad AS. Zinc: role in immunity, oxidative stress and chronic inflammation. Curr Opin Clin Nutr Metab Care 2009;12:646–52.
[20] Fraker PJ, King LE. Reprogramming of the immune system during zinc deficiency. Annu Rev Nutr 2004;24:277–98.
[21] Kahmann L, Uciechowski P, Warmuth S, Malavolta M, Mocchegiani E, Rink L. Effect of improved zinc status on T helper cell activation and TH1/TH2 ratio in healthy elderly individuals. Biogerontology 2006;7:429–35.
[22] Bao B, Prasad AS, Beck FW, Godmere M. Zinc modulates mRNA levels of cytokines. Am J Physiol Endocrinol Metab 2003;285:E1095–102.
[23] von Bulow V, Dubben S, Engelhardt G, Hebel S, Plumakers B, Heine H, et al. Zinc-dependent suppression of TNF-alpha production is mediated by protein kinase A-induced inhibition of Raf-1, I kappa B kinase beta, and NF-kappa B. J Immunol 2007;179:4180–6.
[24] Haase H, Ober-Blobaum JL, Engelhardt G, Hebel S, Heit A, Heine H, et al. Zinc signals are essential for lipopolysaccharide-induced signal transduction in monocytes. J Immunol 2008;181:6491–502.
[25] Dubben S, Honscheid A, Winkler K, Rink L, Haase H. Cellular zinc homeostasis is a regulator in monocyte differentiation of HL-60 cells by 1 alpha,25-dihydroxyvitamin D3. J Leukoc Biol 2010;87:833–44.
[26] Yui S, Nakatani Y, Hunter MJ, Chazin WJ, Yamazaki M. Implication of extracellular zinc exclusion by recombinant human calprotectin (MRP8 and MRP14) from target cells in its apoptosis-inducing activity. Mediators Inflamm 2002;11:165–72.

[27] Wessels I, Fleischer D, Rink L, Uciechowski P. Changes in chromatin structure and methylation of the human interleukin-1beta gene during monopoiesis. Immunology 2010;130:410–7.
[28] Stordeur P, Poulin LF, Craciun L, Zhou L, Schandene L, de Lavareille A, et al. Cytokine mRNA quantification by real-time PCR. J Immunol Methods 2002;259:55–64.
[29] Faneyte IF, Kristel PM, van de Vijver MJ. Determining MDR1/P-glycoprotein expression in breast cancer. Int J Cancer 2001;93:114–22.
[30] Liang MD, Zhang Y, McDevit D, Marecki S, Nikolajczyk BS. The interleukin-1beta gene is transcribed from a poised promoter architecture in monocytes. J Biol Chem 2006;281:9227–37.
[31] Lee JY, Kim NA, Sanford A, Sullivan KE. Histone acetylation and chromatin conformation are regulated separately at the TNF-alpha promoter in monocytes and macrophages. J Leukoc Biol 2003;73:862–71.
[32] Dahl JA, Collas P. Q2ChIP, a quick and quantitative chromatin immunoprecipitation assay, unravels epigenetic dynamics of developmentally regulated genes in human carcinoma cells. Stem Cells 2007;25:1037–46.
[33] Haase H, Hebel S, Engelhardt G, Rink L. Flow cytometric measurement of labile zinc in peripheral blood mononuclear cells. Anal Biochem 2006;352:222–30.
[34] Krezel A, Maret W. Zinc-buffering capacity of a eukaryotic cell at physiological pZn. J Biol Inorg Chem 2006;11:1049–62.
[35] Castro CE. Nutrient effects on DNA and chromatin structure. Annu Rev Nutr 1987;7:407–21.
[36] Barbieri SS, Eligini S, Brambilla M, Tremoli E, Colli S. Reactive oxygen species mediate cyclooxygenase-2 induction during monocyte to macrophage differentiation: critical role of NADPH oxidase. Cardiovasc Res 2003;60:187–97.
[37] Lee SA, Kim EY, Jeon WK, Woo CH, Choe J, Hahn S, et al. The inhibitory effect of raloxifene on lipopolysaccharide-induced nitric oxide production in RAW264.7 cells is mediated through a ROS/p38 MAPK/CREB pathway to the up-regulation of heme oxygenase-1 independent of estrogen receptor. Biochimie 2010;93:168–74.
[38] Clark AR, Dean JL, Saklatvala J. Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. FEBS Lett 2003;546:37–44.
[39] Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol 1997;272:E1002–7.
[40] Haase H, Rink L. The immune system and the impact of zinc during aging. Immun Ageing 2009;6:9.
[41] Lin WW, Chen BC. Distinct PKC isoforms mediate the activation of cPLA2 and adenylyl cyclase by phorbol ester in RAW264.7 macrophages. Br J Pharmacol 1998;125:1601–9.
[42] Cousins RJ, Blanchard RK, Popp MP, Liu L, Cao J, Moore JB, et al. A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. Proc Natl Acad Sci U S A 2003;100:6952–7.
[43] Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM, Ojcius DM. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J Biol Chem 2007;282:2871–9.
[44] Zago MP, Mackenzie GG, Adamo AM, Keen CL, Oteiza PI. Differential modulation of MAP kinases by zinc deficiency in IMR-32 cells: role of H(2)O(2). Antioxid Redox Signal 2005;7:1773–82.
[45] Manthey CL, Wang SW, Kinney SD, Yao Z. SB202190, a selective inhibitor of p38 mitogen-activated protein kinase, is a powerful regulator of LPS-induced mRNAs in monocytes. J Leukoc Biol 1998;64:409–17.
[46] Liuzzi JP, Lichten LA, Rivera S, Blanchard RK, Aydemir TB, Knutson MD, et al. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc Natl Acad Sci U S A 2005;102:6843–8.
[47] Prasad AS, Bao B, Beck FW, Kucuk O, Sarkar FH. Antioxidant effect of zinc in humans. Free Radic Biol Med 2004;37:1182–90.