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Zinc-Dependent Suppression of TNF-α Production Is Mediated by Protein Kinase A-Induced Inhibition of Raf-1, IκB Kinase β, and NF-κB[1]

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Excessive and permanent cytokine production in response to bacterial LPS causes cell and tissue damage, and hence organ failure during sepsis. We have previously demonstrated that zinc treatment prevents LPS-induced TNF-α expression and production in human monocytes by inhibiting cyclic nucleotide phosphodiesterase (PDE) activity and expression, and subsequent elevation of the cyclic nucleotide cGMP. In the present study, we investigated the molecular mechanism by which cGMP signaling affects the LPS-induced signaling cascade to suppress TNF-α transcription and release from monocytes. Zinc-mediated cGMP elevation led to cross activation of protein kinase A. This zinc-induced protein kinase A activation inhibited Raf-1 activity by phosphorylation at serine 259, preventing activation of Raf-1 by phosphorylation of serine 338. By this mechanism, zinc suppressed LPS-induced activation of IκB kinase β (IKKβ) and NF-κB, and subsequent TNF-α production. Our study shows that PDE inhibition by zinc modulates the monocytic immune response by selectively intervening in the Raf-1/IKKβ/NF-κB pathway, which may constitute a common mechanism for the anti-inflammatory action of PDE inhibitors. *The Journal of Immunology,* **2007, 179: 4180–4186.**

Septic shock is a systemic inflammatory response syndrome resulting from a harmful or damaging host response to infection. The outcome of sepsis is determined by excessive and uncontrolled cytokine production, which is mainly mediated by the transcription factor NF-κB in response to bacterial LPS. This disease remains the leading cause of death among critically ill patients, despite many efforts to develop an effective therapy (1). One promising approach is the inhibition of the pathway of LPS-induced NF-κB activation and cytokine secretion.

In addition to its essential role in various immunological functions (2, 3), zinc has a dual effect on the secretion of proinflammatory cytokines. It has been reported to trigger the release of these cytokines by monocytes and monocytic cell lines (4, 5), but suppression of proinflammatory cytokine production has also been found (6, 7). This is explained by a concentration-dependent process in which low zinc doses potentiate the LPS effect, whereas high concentrations inhibit monokine secretion (8, 9). Inhibitory zinc concentrations suppress the monocytic response to LPS by inhibiting cyclic nucleotide phosphodiesterase (PDE)[3] activity and expression. The resulting increase of cellular cGMP suppresses LPS-induced TNF-α and IL-1β production in human monocytes (9), but in which way zinc-induced cGMP affects the signaling events leading to cytokine production is unclear.

The intracellular concentrations of cAMP and cGMP are regulated through synthesis by adenylate and guanylate cyclase, and degradation by PDE. Cyclic nucleotides transduce signals through direct protein binding. The principle intracellular targets of cAMP and cGMP are protein kinase A (PKA) and protein kinase G (PKG), respectively, but high concentrations of cGMP can cross-activate PKA (10). Activation of PKA by cGMP and the cGMP analog 8-Br-cGMP has been studied in human platelets and in guinea pig tracheal smooth muscle cells (11, 12). Human monocytes express two types of PKA, type I and II. Each kinase is a tetramer of two regulatory domains bound to two catalytic domains. Upon cooperative binding of four cyclic nucleotide molecules, the regulatory subunits disengage from the catalytic subunits, which are then free to phosphorylate specific targets (13). In human monocytes, cAMP-elevating agents suppress the release of TNF-α, and this effect is antagonized by inhibition of type II PKA (14).

The mammalian Raf protein kinase gene family consists of Raf-1, A-Raf, and B-Raf which share three domains, conserved region (CR) 1, CR2, and CR3 (15). Activation of Raf-1 is involved in LPS-stimulated TNF-α synthesis in monocytes and macrophages (16, 17). Active Raf-1 phosphorylates and activates MEK, which in turn activates ERK (18). Raf-1 activation induces expression of reporter genes driven by the NF-κB promoter (15). PKA phoshorylates serine 259 in the CR2 regulatory domain of Raf-1, hereby negatively regulating its activity (19). On the molecular level, binding of the chaperonin 14-3-3 to inactive Raf-1 stabilizes the inactive conformation, and dephosphorylation of serine 259 by phosphatase PP2A encourages dissociation of 14-3-3 and allows activation of Raf-1 by phosphorylation at serine 338 in the kinase domain CR3 (18, 20).

The aim of this study was to investigate how zinc-mediated elevation of cellular cGMP modulates signal transduction to suppress LPS-induced TNF-α synthesis. It was found that zinc-mediated cGMP elevation cross-activated PKA, leading to inhibitory

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[3] Abbreviations used in this paper: PDE, cyclic nucleotide phosphodiesterase; sGC, soluble guanylate cyclase; PKA, protein kinase A; PKG, protein kinase G; CR, conserved region; IKKβ, IκB kinase β; RA, rheumatoid arthritis.

phosphorylation of Raf-1 at serine 259. Hereby, PKA intervened in the Raf-1 pathway and inhibited NF-κB translocation and TNF-α production in LPS-stimulated monocytes.

Materials and Methods

Media and reagents

RPMI 1640 medium, sodium pyruvate, L-glutamine, nonessential amino acids, penicillin, streptomycin, and PBS were purchased from Cambrex. FCS was obtained from PAA Laboratories. cAMP, cGMP, 8-CPT-cAMP, DB-cAMP, Rp-8-pCPT-cGMPS, Rp-8-CPT-cAMPS, and Sp-8-CPT-cAMPS were purchased from Biolog. LPS *Escherichia coli* serotype O111: B4, 6-(phenylamino)-5,8-quinolinedione (LY83583), sodium pyrithione, protease inhibitor mixture, 3-isobutyl-1-methylxanthin, and Pefablock were from Sigma-Aldrich. Raf-1 kinase inhibitor I was purchased from Calbiochem.

Cell culture

Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids, and 1 mM sodium pyruvate (Mono Mac1) or DMEM medium, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 400 μg/ml G418 (RAW 264.7).

Isolation and culture of PBMC and monocytes

PBMC were isolated from heparinized peripheral venous blood from healthy donors by centrifugation over Ficoll-Hypaque, washed three times with PBS, and resuspended in RPMI 1640 medium (containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine). For enrichment of monocytes, PBMC were seeded on glass coverslips at a density of 2×10^6 cells/ml and were incubated overnight at 37°C and 5% CO_2 using 10% donor serum instead of FCS. Nonadherent cells were removed by washing with medium at 37°C.

Protein kinase assays

Mono Mac1 cells (5×10^6) were treated with zinc and pyrithione, LPS, or LY83583 for 6 h as indicated in the figure legends. Cells were washed two times with ice-cold PBS and lysed by the addition of 375 μl of lysis buffer (50 mM HEPES, 100 mM NaCl, 50 mM sodium fluoride, 5 mM β-glycerophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% (v/v) Triton X-100, 0.1 mM 3-isobutyl-1-methylxanthin, and 5 mM protease inhibitor mixture) for 10 min on ice. The protein concentration was determined by Bradford assay (Bio-Rad) after centrifugation for 10 min at 13,000 × *g*. For measurement of PKA activity, 10 μl of 5-fold kinase buffer (250 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$) was added to 0.1 mM ATP, 300 μM of the specific PKA substrate KEMPtide (21) (Calbiochem), 100 μM Rp-8-pCPT-cGMPS (specific PKG inhibitor), 5 μCi [γ-^{32}P]ATP (sp. act., 3000 Ci/mmol), and 50 μg protein in a final volume of 50 μl. For the PKG activity assay, the specific PKG substrate BPDEtide (22) (300 μM; Calbiochem) was used in the presence of the specific PKA inhibitor Rp-8-CPT-cAMPS (100 μM). The samples were incubated for 30 min at 30°C. The reaction was stopped by pipetting the sample onto P81 phosphocellulose paper (Whatman). The filter papers were washed three times in 75 mM orthophosphoric acid and in acetone. Filter papers were dried, solubilized in scintillation fluid (Picofluor 40; PerkinElmer), and the amount of ^{32}P bound to the peptides was determined using a beta counter (Packard Tri-Carb 2100TR). In Mono Mac1 cells, no cGMP-stimulated PKG activity was detected with this method, but the functionality of the assay was confirmed in HEK293T cells.

Immunoblotting

Mono Mac1 cells were lysed in sample buffer (0.5 M Tris-HCl, 4% (v/v) glycerol, 2% (w/v ratio) SDS, 0.01% (w/v ratio) bromophenol blue, 1% (v/v) 2-ME), sonicated for 3 s and boiled for 5 min at 95°C. Aliquots (20 μl/lane) containing either lysate of 4×10^5 cells (detection of Raf-1 and IKK) or 25 μg protein (detection of PKA_c) were separated electrophoretically on polyacrylamide gels. After electrotransfer to nitrocellulose, the membranes were blocked with 5% dry milk in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) for 1 h, rinsed with TBS-T, and incubated with Abs against Raf-1 phosphorylated either on serine 338 or serine 259, total Raf-1, IKKαβ phosphorylated on serines 176 and 180, total IKKβ (all from Cell Signaling Technology), or the catalytic subunit of PKA (BD Biosciences) for 1 h. Membranes were incubated with HRP-linked anti-rabbit IgG (for Raf-1 and IKK; Cell Signaling Technology) or anti-mouse IgG (for PKA; Cell Signaling Technology) Abs for 1 h. Immunocomplexes were detected with a luminol chemiluminescent substrate (LumiGlo; Cell Signaling Technology) and exposed to Kodak X-OMAT UV film.

Preparation of nuclear extracts

For extraction of nuclear proteins, 2×10^7 Mono Mac1 cells were seeded in 25-cm^2 plastic flasks 24 h before treatment with zinc and LPS for 1 h as indicated in the figure legends. Mono Mac1 cells were washed twice with ice-cold PBS containing 100 μM Na_3VO_4. Cells were harvested in 1 ml of hypotonic buffer (10 mM HEPES-KOH (pH 7.6), 2 mM $MgCl_2$, 15 mM KCl, 100 μM Na_3VO_4, 0.5 mM protease inhibitor mixture, 0.5 mM Pefablock, 0.1 mM EDTA, and 1 mM DTT) and lysed for 10 min at 4°C. After centrifugation (700 × *g* at 4°C) for 5 min, supernatants were discarded. The pellets were resuspended in 20 mM HEPES-KOH (pH 7.6) containing 420 mM NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 100 μM Na_3VO_4, 0.5 mM protease inhibitor mixture, 0.5 mM Pefablock, and 0.5 mM DTT. After 20 min incubation at 4°C and centrifugation at 16,000 × *g* for 10 min, nuclear extracts were stored at −80°C until further processing. Protein concentrations of nuclear extracts were measured with the Bradford protein assay (Bio-Rad).

EMSAs

Double-stranded oligonucleotides containing the NF-κB consensus sequence were generated by annealing two single-stranded oligonucleotides: 5′-TGC CTG GGA AAG TCC CCT CA-3′ and 5′-AGT TGA GGG GAC TTT CCC AGG-3′. After labeling of oligonucleotide probes with [α-^{32}P]dATP (10 mCi/ml, 3000 Ci/mmol) using Klenow enzyme (Fermentas), the unincorporated label was removed using the QiaQuick nucleotide removal kit (Qiagen). For each bandshift reaction, 20 μg of nuclear protein extract were incubated with 100 fmol of probe in bandshift buffer (10 mM Tris-HCL (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5% (v/v) glycerol, 10 mM DTT), and 1 μg poly(dI:dC) (Sigma-Aldrich) in a final volume of 20 μl for 30 min at room temperature. All protein DNA complexes were resolved on a 6% polyacrylamide gel containing 7.5% glycerol in 0.25-fold Tris-borate-EDTA buffer (20 mM Tris-HCl (pH 8.0), 20 mM boric acid, and 0.5 mM EDTA) and electrophoresed at 200 V for 2.5 h. Gels were dried at 70°C for 2 h, exposed to phosphor screens overnight, and scanned with a Molecular Imager FX (Bio-Rad).

NF-κB immunofluorescence

Cells were fixed for 5 min with ice-cold methanol, air dried, and incubated with blocking serum (10% FCS in PBS) for 20 min at room temperature. Cells were then treated for 1 h with polyclonal IgG against the NF-κB p65 subunit (sc-109; Santa Cruz Biotechology) and 45 min with FITC-labeled goat anti-rabbit secondary Ab. For the last 10 min, Hoechst 33258 (final concentration 150 μM) was added, and the fluorescence was analyzed on a Zeiss Axioskop fluorescence microscope.

Measurement of cellular cAMP and cGMP

For measurement of cellular cAMP and cGMP, Mono Mac1 cells (cAMP 2×10^6cells/ml, cGMP 1.25×10^6/ml) were seeded in polypropylene-tubes and incubated for 6 h at 37°C and 5% CO_2 and analyzed as described previously (9). The cellular cAMP level was detected using the Delfia cAMP Kit (PerkinElmer), a time-resolved fluoroimmuno assay based on europium dissociative fluorescence enhancement. Cellular cGMP was analyzed with the cGMP enzyme immunoassay (IBL).

Quantification of cytokines

Cells (5×10^5 cells/ml) were seeded in 12-well tissue culture dishes 24 h before stimulation. After the incubations, culture supernatants were harvested and stored at −80°C. For quantification of TNF-α, the enzyme immunoassay OptEIA from BD Pharmingen was used. Results were measured with an ELISA plate reader (Sunrise; Tecan).

TNF promoter reporter assay

Murine RAW 264.7 macrophages stably transfected with the human TNF promoter in front of the gene for EGFP were plated at a density of 2×10^5 in normal culture medium 24 h before the incubations. After the incubation, cells were cultured for another 24 h and the EGFP fluorescence was measured by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Cellular survival was confirmed by costaining with propidium iodide.

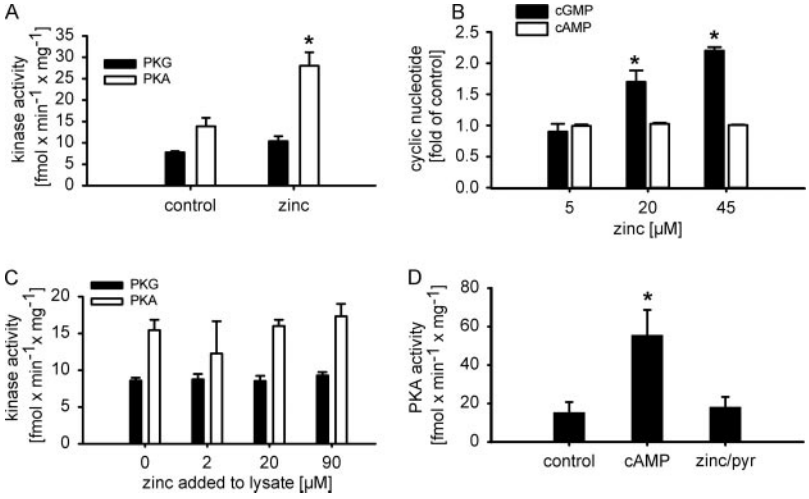

FIGURE 1. Activation of PKA and PKG by zinc ions. *A*, Mono Mac1 cells were treated with zinc sulfate (45 μM) and pyrithione (50 μM) for 6 h, and PKA and PKG activities were measured in the lysate as described under *Materials and Methods*. *B*, Mono Mac1 cells were treated with 5 μM, 20 μM, or 45 μM $ZnSO_4$ in the presence of 50 μM pyrithione for 6 h. Cellular lysates were analyzed by cGMP enzyme immunoassay or by cAMP fluoroimmunoassay. *C* and *D*, Mono Mac1 lysate was incubated for 30 min with 2 μM, 20 μM, or 90 μM $ZnSO_4$ (*C*) or with 1 μM cAMP or 45 μM zinc together with 50 μM pyrithione (*D*), and kinase activities were measured as under *A*. All values are expressed as mean ± SEM from at least three independent experiments. Statistically significant ($p < 0.05$) changes of PKA activity (*A, C, and D*) or cGMP and cAMP concentrations (*B*) compared with control values are indicated (∗).

Statistical analysis

Statistical significance of experimental results was calculated by an unpaired, two-sided Student's *t* test.

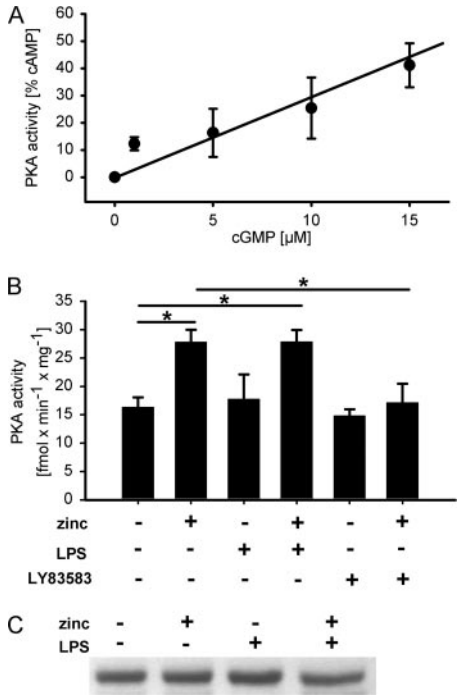

FIGURE 2. Zinc induces cross activation of PKA by cGMP. *A*, Mono Mac1 lysate was incubated with different concentrations of cGMP and PKA activity was measured as in Fig. 1. Values shown are means from $n = 5$ independent experiments ± SEM, presented as percent PKA activity in the presence of 1 μM cAMP. The line indicates the linear regression curve. *B*, Mono Mac1 cells were stimulated with zinc sulfate (45 μM) and pyrithione (50 μM) for 6 h alone or in the presence of LPS (250 ng/ml) or LY83583 (1 μM, 30 min before zinc/pyrithione). Cells were lysed and PKA activity analyzed by protein kinase assay. Values are expressed as the mean ± SEM from at least three independent experiments. Statistically significant ($p < 0.05$) PKA activity changes are indicated (∗). *C*, Mono Mac1 cells were incubated with zinc sulfate (45 μM)/pyrithione (50 μM) and/or LPS (250 ng/ml) for 6 h. The lysate was analyzed by Western blot. The catalytic subunit of PKA was detected at 40 kDa. Data are representative of three independent experiments.

Results

Activation of PKA by zinc ions

Zinc suppresses TNF-α release from human monocytes by inhibition of PDE (9). This implies an involvement of PKG or PKA. Hence, we investigated the activation of these kinases following zinc treatment using specific PKA and PKG activity assays. Incubation of Mono Mac1 cells with 45 μM zinc and 50 μM pyrithione for 6 h significantly increased PKA activity, whereas PKG was not affected (Fig. 1*A*). Although this effect points toward an increase of cAMP and subsequent PKA activation, previous observations

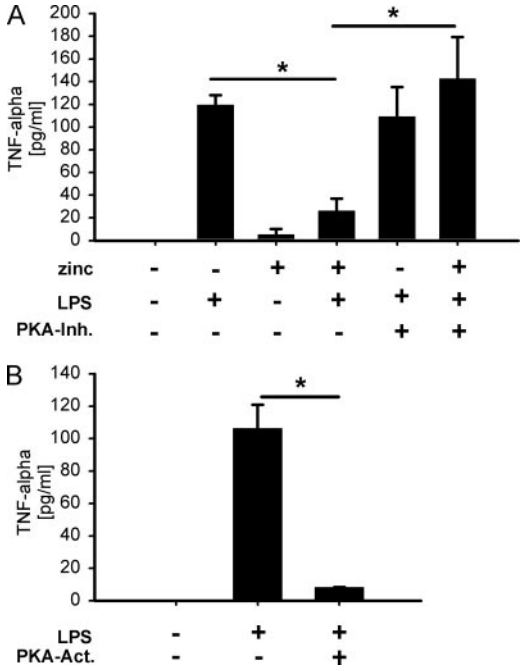

FIGURE 3. Reversal of zinc-mediated TNF-α suppression by inhibition of PKA. Mono Mac1 cells were preincubated with the specific PKA inhibitor Rp-8-CPT-cAMPS (200 μM) (*A*), or the specific PKA activator Sp-8-CPT-cAMPS (200 μM) (*B*) for 30 min before treatment with LPS (250 ng/ml) and/or zinc sulfate (20 μM) and pyrithione (50 μM). The TNF-α content of the supernatant was analyzed by ELISA after 4 h of stimulation. Values are expressed as mean ± SEM from at least three independent experiments. Statistically significant ($p < 0.05$) changes compared with cells stimulated with zinc sulfate/pyrithione and LPS (*A*) or LPS alone (*B*) are indicated (∗).

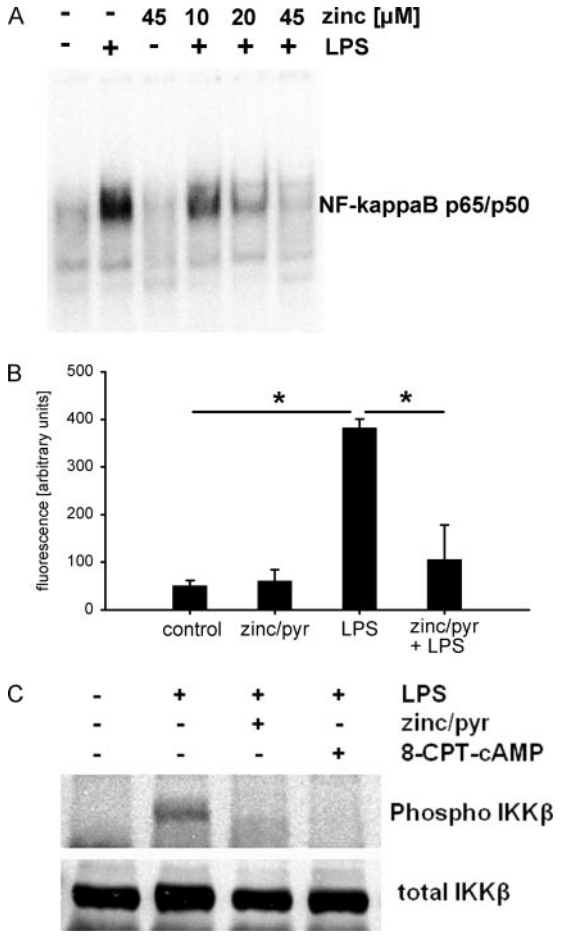

FIGURE 4. Effect of zinc on LPS-induced transcriptional activation. *A*, Effect of zinc on LPS-induced NF-κB activation. Mono Mac1 cells were stimulated for 1 h and nuclear extracts were analyzed by EMSA with an NF-κB consensus site, either after treatment with LPS (250 ng/ml) and 10 μM, 20 μM, or 45 μM zinc sulfate/pyrithione (50 μM). Data are representative of three independent experiments. Unbound probe at the bottom of the gel was cut off. *B*, RAW 264.7 stably transfected with EGFP under the control of the human TNF-α promoter were stimulated for 24 h with LPS (250 ng/ml) and zinc/pyrithione (15 μM/10 μM) as indicated and the resulting fluorescence was analyzed by flow cytometry. Data are shown as means from $n = 4$ independent experiments ± SEM. *C*, Mono Mac1 cells were stimulated for 30 min with LPS (250 ng/ml) alone, or after preincubation with zinc sulfate (25 μM) and pyrithione (50 μM) or the PKA activator 8-CPT-cAMP (2 mM), and phosphorylation and total protein content of IKKβ was analyzed by Western blot. Data shown are representative of three independent experiments.

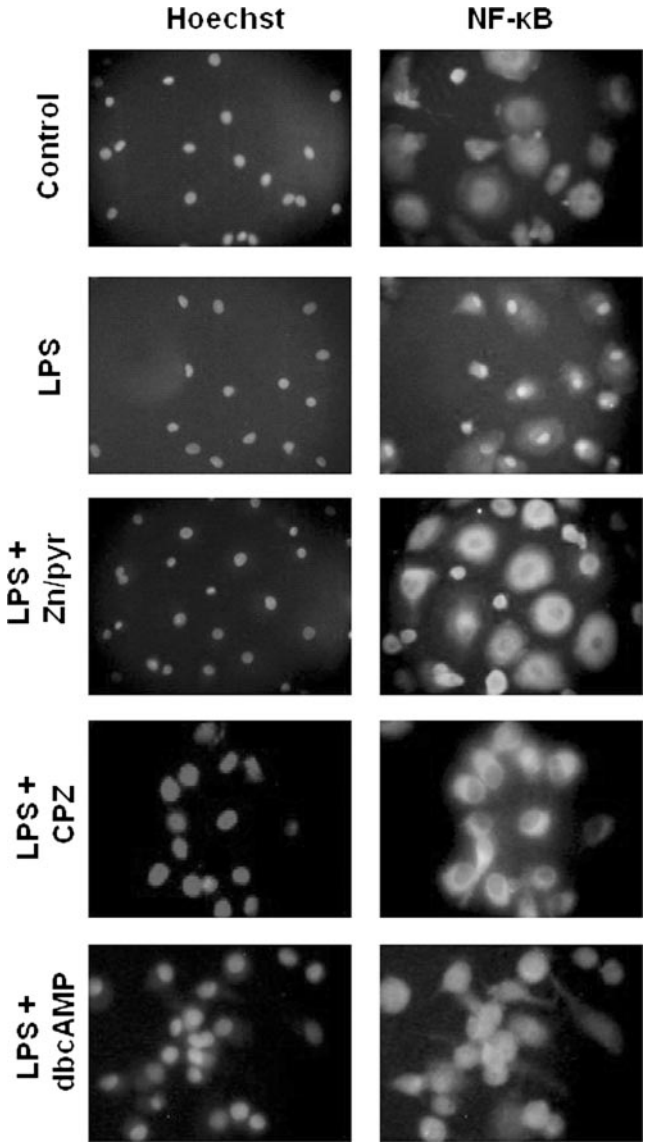

FIGURE 5. Effect of zinc, chlorpromazine, and DB-cAMP on NF-κB nuclear translocation. Primary human monocytes were stimulated with LPS (250 ng/ml) alone, or after preincubation with zinc sulfate (25 μM) and pyrithione (50 μM), the PDE-1 inhibitor chlorpromazine (CPZ, 100 μM), or the PKA activator DB-cAMP (1 mM). NF-κB was stained with polyclonal IgG against the p65 subunit and a FITC-labeled secondary Ab and nuclei with Hoechst 33258. Pictures shown are representative of at least three independent experiments.

indicated an effect of zinc solely on cGMP (9). Therefore, concentrations of cAMP and cGMP were measured in Mono Mac1 cells after zinc treatment, confirming that zinc causes a dose dependent increase of cellular cGMP but not cAMP (Fig. 1*B*). A direct activation of PKA by zinc can be excluded, because neither kinase was activated by addition of zinc to the lysate (Fig. 1*C*), and PKA was also not activated by a combination of zinc and the ionophor pyrithione (Fig. 1*D*).

Role of cGMP in zinc-induced PKA activation

Incubation of Mono Mac1 cell lysate with different concentrations of cGMP resulted in a concentration-dependent activation of PKA (Fig. 2*A*). Although cGMP did not activate PKA as strongly as cAMP, this indicates that the increased PKA activity after zinc stimulation could be mediated through cross activation by cGMP. The absence of a significant effect of zinc-treatment on PKG activity is explained by a lack of cGMP-dependent PKG activity in the lysate of Mono Mac1 cells (data not shown).

To ensure that the modulation of PKA activity by zinc also occurs during stimulation with LPS, Mono Mac1 cells were treated with zinc (45 μM) and pyrithione (50 μM) in the absence or presence of LPS (250 ng/ml). LPS neither affects PKA activity in resting cells, nor does it alter the effect of zinc (Fig. 2*B*). When the soluble GC (sGC) inhibitor LY83583 was added together with zinc to block cGMP synthesis, the zinc-induced increase of PKA activity was suppressed (Fig. 2*B*), confirming that the activation of PKA requires cGMP. A regulation of PKA by zinc or LPS on the transcriptional level was excluded, because the amount of the PKA catalytic subunit was unchanged by 6 h incubation with zinc (45 μM) and pyrithione (50 μM) or LPS (250 ng/ml) (Fig. 2*C*).

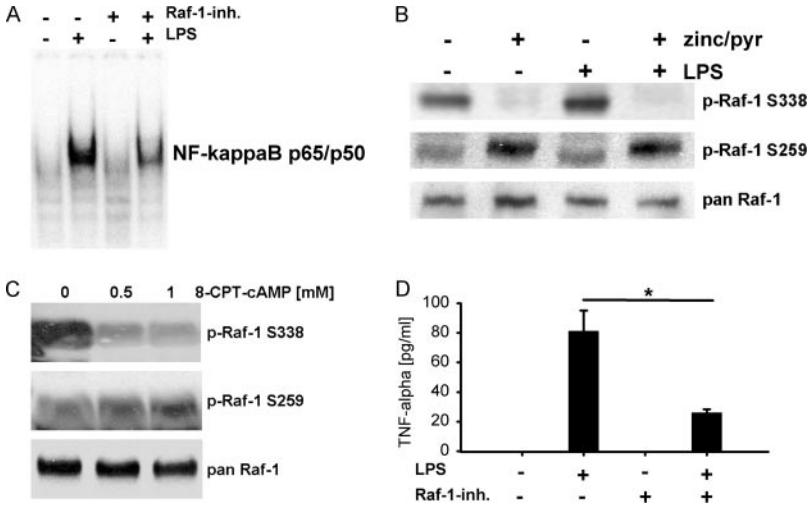

FIGURE 6. Zinc-induced alteration of Raf-1 activation. *A*, Mono Mac1 cells were stimulated for 1 h, and nuclear extracts were analyzed by EMSA after treatment with Raf-1 inhibitor (1 μM, 30 min before LPS stimulation) and LPS (250 ng/ml). Unbound probe at the bottom of the gel was cut off. *B* and *C*, Raf-1 phosphorylation was analyzed by Western blot using phosphorylation state specific Abs as indicated. Raf-1 phosphorylation and protein was detected at 74 kDa. Mono Mac1 cells were incubated for 30 min either with LPS (250 ng/ml) and/or zinc sulfate (45 μM)/pyrithione (50 μM) (*B*), or with the PKA activator 8-CPT-cAMP (*C*). Data are representative of three independent experiments (*A–C*). *D*, Mono Mac1 cells were incubated with Raf-1 inhibitor (1 μM, 30 min before LPS stimulation) and LPS (250 ng/ml) for 4 h. TNF-α content of the supernatants was analyzed by ELISA 4 h after addition of LPS. Values are expressed as mean ± SEM from three independent experiments performed in duplicates. Statistically significant ($p < 0.05$) changes in TNF-α values compared with LPS alone are indicated (∗).

Role of PKA in zinc-mediated TNF-α suppression

To investigate the role of PKA in zinc-mediated suppression of TNF-α release, we used the specific PKA inhibitor Rp-8-CPT-cAMPS and the specific PKA activator Sp-8-CPT-cAMPS. The inhibitory effect of zinc on LPS-induced TNF-α secretion was antagonized by preincubation with Rp-8-CPT-cAMPS (Fig. 3*A*), and LPS-induced TNF-α production was suppressed by Sp-8-CPT-cAMPS (Fig. 3*B*). These results confirm the involvement of PKA in the zinc-mediated suppression, and demonstrate that PKA activation is sufficient to inhibit LPS-induced TNF-α production. When the effect of PKA on IL-1β secretion was investigated, cytokine secretion was not completely restored under the same conditions (data not shown), indicating that the inhibitory effect of zinc on IL-1β is not solely mediated by PKA.

LPS-induced NF-κB activation is antagonized by zinc

To examine the role of zinc-mediated PKA activation on downstream signaling, the influence on NF-κB was investigated by EMSAs with a NF-κB consensus sequence. Binding of nuclear proteins from Mono Mac1 cells to this κB site was examined 1 h after LPS stimulation. Zinc decreased the LPS-induced activation of p50/p65 in a concentration-dependent manner (Fig. 4*A*). The identity of this complex has previously been confirmed by Ab supershift experiments (23). A NF-κB transcription factor assay confirmed the EMSA results, showing that the level of LPS-induced p50 DNA binding was significantly reduced by zinc (data not shown). Zinc can also inhibit the activation of the TNF promoter in intact cells, which has been confirmed in the RAW 264.7 murine monocyte/macrophage cell line stably transfected with EGFP under the control of the human TNF promoter (Fig. 4*B*). The inhibition is not due to a direct effect of zinc and PKA on NF-κB DNA binding, but on signal transduction upstream of NF-κB. Zinc/pyrithione, as well as the PKA activator 8-CPT-cAMP were able to block the LPS-induced activating phosphorylation of IKKβ in Mono Mac1 cells (Fig. 4*C*), and the nuclear translocation of NF-κB was inhibited by zinc/pyrithione, chlorpromazine (an inhibitor of the cGMP degrading PDE-1), and the PKA activator DB-cAMP in primary human monocytes (Fig. 5).

LPS-induced NF-κB activation is antagonized by Raf-1 inhibition

Raf-1 is involved in the LPS-induced activation of TNF-α synthesis in human monocytes (16). Because PKA is known to inhibit Raf-1 activity (18), we tested whether inhibition of Raf-1 could block LPS-induced NF-κB signaling. Incubation of Mono Mac1 cells with the Raf-1 kinase inhibitor I (1 μM) resulted in a decreased activation of NF-κB by LPS (Fig. 6*A*). LPS causes the activation of Raf-1 by phosphorylation (24), which could be antagonized by zinc treatment, because phosphorylation of serine 259 by PKA can inhibit Raf-1 activation (18). Therefore, we investigated whether zinc-induced activation of PKA modulates Raf-1 phosphorylation. Western blot experiments with Mono Mac1 cells showed that Raf-1 was phosphorylated at serine 338 in control cells and LPS-stimulated cells, but the activating phosphorylation at serine 338 was reduced by incubation with zinc and pyrithione (Fig. 6*B*). A constitutive phosphorylation of Raf-1 at serine 338 in resting cells is not unusual; it has previously been described in murine dendritic DC2.4 cells (25). Contrary to the effects observed with serine 338, the inhibitory Raf-1 phosphorylation at serine 259 increased when cells were treated with zinc (45 μM) and pyrithione (50 μM) alone and after costimulation with LPS (Fig. 6*B*), confirming a zinc-induced switch from activating to inhibiting Raf-1 phosphorylation. The presence of equal amounts of Raf-1 protein in all samples was confirmed with an Ab against total Raf-1 (Fig. 6*B*). The effect of PKA on Raf-1 phosphorylation was confirmed with the PKA activator 8-CPT-cAMP. Treatment of Mono Mac1 resulted in decreased phosphorylation of serine 338, and an increase in serine 259 phosphorylation (Fig. 6*C*). To ensure that Raf-1 inhibition is sufficient to block TNF-α secretion in response to stimulation with LPS, Mono Mac1 cells were treated with a Raf inhibitor and TNF-α was measured by ELISA in the supernatant, confirming that inhibition of Raf-1 significantly diminishes LPS-induced TNF-α synthesis (Fig. 6*D*).

Discussion

Dependent on the concentration, zinc supplementation has either stimulatory or inhibitory effects on cytokine secretion by monocytes (4–9). However, in which way zinc exerts these effects on the molecular level is poorly understood. We have previously demonstrated that zinc treatment of human monocytes suppresses LPS-induced TNF-α expression and release by inhibition of PDE activity and expression, resulting in cGMP accumulation (9). In the present study, we investigated the mechanism by which zinc-mediated elevation of cellular cGMP affects signal transduction to suppress TNF-α synthesis in monocytes. Inhibition of PDE increases cellular cGMP, which cross-activates PKA. This kinase intervenes in LPS-induced signaling by affecting the Raf-1 pathway in monocytic cells, leading to a decrease in NF-κB activation and TNF-α transcription. Activation of PKG by cGMP was not observed, due to a lack of cGMP stimulated PKG activity in Mono Mac1 cells, and PKA activation by cAMP can be excluded because cAMP was not increased by zinc ions. The latter effect might be due to an inhibition of cAMP synthesis by zinc (26), because zinc ions can inhibit the GTPase activity of the stimulatory GTP binding protein, a regulator of adenylate cyclase (27). It is not known whether zinc inhibits guanylate cyclase in a similar fashion, but cGMP is also synthesized by the G protein-independent NO-regulated sGC, whose inhibition by LY83583 abrogated the effects of zinc in our experiments.

Three phosphorylation sites in Raf-1, serine 43 (28), 259 (19, 20), and 621 (29) are phosphorylated by PKA. Raf-1 inhibition is mainly mediated through serine 259 phosphorylation, and Raf-1 is the sole target for PKA-mediated inhibition of ERK and ERK-induced gene expression (19). It was demonstrated that PKA can decrease the activating phosphorylation of Raf-1 at serine 338 (19). Dephosphorylation of serine 259 is mediated by phosphatase PP2A in macrophages (30) and is a key step in Raf-1 activation, regulating its binding to its substrate MEK (20). A different mechanism for zinc-mediated down-regulation of Raf-1 activity has been found in *Xenopus* oocytes. In this study, the kinase activity of Raf-1 is promoted by binding to the zinc export protein ZnT-1, and the addition of zinc inhibits the binding of Raf-1 to ZnT-1 (31). Such a mechanism of Raf-1 inhibition by zinc can be excluded in monocytes, because inhibition of sGC (9) or PKA antagonized the inhibitory effect of zinc on TNF-α secretion, providing evidence for an involvement of cyclic nucleotide signaling.

From our results, it seems clear that zinc-mediated cross-activation of PKA by cGMP does not interfere directly with the interaction between NF-κB and DNA, but acts upstream, leading to inhibition of the LPS-induced phosphorylation of the positive regulator IKK, and preventing nuclear translocation. In addition, in our experimental system, there was no direct effect of up to 1 mM zinc on NF-κB DNA binding (data not shown). However, it remains to be investigated in more detail in which way Raf-1 affects TNF-α transcription. Raf is involved in the activation of several transcription factors, not only NF-κB, but also CREB, Ets, AP-1, and c-Myc (15), and, in addition to three binding sites for NF-κB, binding sites for Ets and AP-1 have also been described for the TNF promoter (32). The classical target for Raf-1 is the MAPK kinase MEK, which in turn activates Erk1/2. However, NF-κB activation by Raf is mediated by the MAPK/ERK kinase kinase-1, and subsequent phosphorylation of IKKβ (15). We were able to demonstrate an effect of zinc and PKA activation on LPS-induced IKKβ phosphorylation, strongly suggesting that this pathway is involved. However, it cannot be excluded that other pathways do also play a role.

Several animal studies demonstrated that zinc can protect against the inflammatory TNF-α response induced by endotoxin (7, 33, 34). Pretreatment with zinc protects against lethality in endotoxin-challenged mice (33) and significantly decreases TNF-α plasma levels in a porcine model of septic shock (34). Moreover, pretreatment with zinc attenuates LPS-induced TNF-α release from Kupffer cells due to suppression of NF-κB activation, thereby preventing hepatotoxicity in mice (7). Our study suggests a molecular mechanism by which zinc treatment exerts its protective effect. The inhibition of cytokine production by zinc makes this ion a promising therapeutic agent that could be used to suppress systemic inflammation or even inflammatory diseases in general. However, administration of zinc protects against septic shock in endotoxin-challenged mice only within a narrow concentration range (33). Therefore, investigations of the dose response have to be done, because zinc can have either pro- or anti-inflammatory effects, which may lead to no effect or even undesired consequences in case the optimal range is not met. In addition, the time of administration should be taken into consideration. Pretreatment of zinc can protect against mortality, but simultaneous administration of zinc and LPS usually increases TNF-α plasma levels, resulting in aggravated inflammation and higher mortality (33–35).

PKA mediated serine 259 phosphorylation and subsequent inhibition of Raf-1 could be a common molecular mechanism shared by zinc and other pharmacological substances that suppress inflammatory cytokine production. Not only the cGMP analogues DB-cGMP and 8-Br-cGMP were found to attenuate LPS-induced TNF-α production in primary monocytes and murine bone marrow-derived macrophages (36, 37), but also pharmacological inhibitors of phosphodiesterase (PDE) are traded as potential anti-inflammatory and immunomodulatory agents to suppress synthesis of proinflammatory cytokines. Therapeutic administration of the unspecific PDE inhibitor pentoxifylline, capable to block the degradation of both cAMP and cGMP, has significant effects on reducing TNF-α plasma levels after 24 h in patients suffering from septic shock (38). Suggested therapeutic targets for PDE-4 inhibitors are several chronic diseases, including asthma, chronic obstructive pulmonary disease, rheumatoid arthritis (RA), and diabetes (39, 40). The specific PDE-4 inhibitor rolipram and the cAMP elevating agent PGE_2 were demonstrated to suppress the proinflammatory cytokine TNF-α by cAMP-induced PKA activation (14). It seems likely that these effects also involve PKA-mediated Raf-1 inactivation, comparable to the actions of zinc. Many of the chronic diseases discussed as therapeutic targets for PDE inhibitors, including asthma (41), chronic obstructive pulmonary disease (42), RA (43), and diabetes (44), are associated with reduced serum zinc levels. Furthermore, the serum zinc level in RA patients is negatively correlated with TNF-α and IL-1β serum levels and zinc supplementation attenuates several symptoms of RA (42). TNF-α and IL-1β are also involved in β cell destruction in diabetes mellitus (44) and supplementation of zinc protects against spontaneous and streptozotocin-induced diabetes in mice (45). A therapeutic administration of zinc may be a tool to modulate PKA activity and thereby the generation of proinflammatory cytokines in chronic inflammatory diseases and septic shock. Zinc is a potential immune modulatory agent, and our results identify one molecular mechanism by which zinc treatment interacts with immune cells, contributing to our understanding of the complex interactions between zinc and the immune system.

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Disclosures

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