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Attenuation of interferon-γ mRNA expression in activated Jurkat T cells by exogenous zinc via down-regulation of the calcium-independent PKC–AP-1 signaling pathway

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ABSTRACT

Zinc is known to modulate a wide variety of cellular functions including anti-inflammatory responses. We examined the intracellular signaling pathways that contribute to the regulation of interferon-gamma (IFN-γ) by zinc in activated human Jurkat T cells. Zinc significantly reduced IFN-γ expression and activator protein-1 (AP-1) signaling in cells activated by phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA) without affecting cell viability. Moreover, partial inhibition of AP-1 activity by SP600125, a c-Jun N-terminal kinase (JNK) inhibitor, resulted in marked reduction of IFN-γ transcription. We also found that this inhibitory effect of zinc on AP-1 signaling was abolished by treatment with rottlerin, a selective inhibitor of calcium-independent protein kinase C (PKC). These results suggest a novel target of zinc in the calcium-independent protein kinase C–AP-1 pathway to regulate endogenous IFN-γ gene expression in activated T cells.
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Introduction

The pathophysiological mechanism of atherosclerosis was recently defined as a chronic vascular inflammatory disorder (Libby, 2002). In the atherosclerotic plaque, various T helper type-1 (Th1) cytokines are produced by vascular endothelial cells, smooth muscle cells, macrophages, and lymphocytes. In particular, a potent pro-inflammatory cytokine, interferon-gamma (IFN-γ), is secreted by activated lymphocytes and enhances Th1-type inflammation in the lesion (Methe et al., 2005; Ranjbaran et al., 2007). Therefore, the regulation of IFN-γ expression in Th1 lymphocytes is considered to be an important therapeutic target for cardiovascular disease.

Zinc is a ubiquitous and essential trace element involved in important biological functions, and was recently demonstrated to act as a novel second messenger (Yamasaki et al., 2007). Furthermore, a human bioinformatic study has revealed at least 1684 proteins as zinc-binding proteins (Andreini et al., 2006), suggesting that zinc deficiency may be involved in a variety of disorders and significant pathological states such as diarrhea and pneumonia (King and Cousins, 2005). A recent epidemiological study revealed that zinc deficiency was responsible for 4.4% of childhood deaths, 14.4% of diarrhea deaths and 6.7% of pneumonia deaths of children between the ages of 6 months to 5 years in developing countries (Fischer Walker et al., in press). Thus, the prevention of zinc deficiency is especially important for growing children with immature immune systems. There are some conflicting reports on the influence of zinc deficiency on immune function. While zinc deficiency is known to suppress Th1 activation (Prasad, 2000; Bao et al., 2003), it is also thought to be a risk factor for cardiovascular disease (Reiterer et al., 2005; Lee et al., 2005). In fact, human atherosclerotic plaques of the abdominal aorta were reported to contain low concentrations of zinc (Vlad et al., 1994). However, the relationship between zinc and Th1-type inflammation remains unclear. It is important to elucidate the precise effect of zinc on the expression of Th1-cytokines, for the regulation of inflammatory diseases. Exogenous zinc was recently reported to inhibit the activities of nuclear factor kappa B (NFκB) and nuclear factor of activated T cells (NFAT), as well as suppress expression of the Th1-type cytokine IL-2 (Takahashi et al., 2003; Tanaka et al., 2005; Bao et al., 2006).

In the present study, we evaluated the effect of zinc on reducing IFN-γ expression in activated human Jurkat T cells. To reveal the mechanism of zinc function in transcriptional regulation, we assessed the activities of transcription factors involved in enhancing inflammation.

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Materials and methods

Cells and reagents

The human T cell lymphoma cell line Jurkat E6.1 (Dainippon Sumitomo Phama) was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The following reagents were purchased: Phorbol 12-myristate 13-acetate (PMA), Gö-6976, and rottlerin (Calbiochem); leucoagglutinin-L (PHA) (Sigma-Aldrich); Lipofectamine™2000 and Superscript™ II (Invitrogen); ISOGEN and DNase (Nippongene); β-D-galactopyranoside, Transcriptor First Strand cDNA Synthesis Kit, and LightCycler® FastStart DNA Master ^{PLUS} SYBR Green I (Roche); 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Dojindo); SP600125 (BIOMOL International L.P.); all vectors and Great EscAPe™ SEAP Chemiluminescence Fluorescence Detection Kit (Clontech); Luciferase Assay System and Reporter lysis buffer (Promega). All primers were synthesized by Nippon EGT. Other reagents were obtained from Wako Pure Chemicals.

Experimental designs

The cells were activated for 6 h in all IFN-γ expression experiments excluding those used for the time course study. To achieve a T cell hyperactivating condition and confirm the time course of IFN-γ expression, cells were stimulated by various concentrations of PHA with or without 200 ng/ml PMA. To evaluate the influence on IFN-γ expression, cells were stimulated with 50 µg/ml PHA and 200 ng/ml PMA in the presence or absence of 50 µM zinc acetate. The effect of zinc on IFN-γ expression was evaluated 6 h after stimulation, and cell viability during the IFN-γ induction was also confirmed. Effects of zinc on the activities of AP-1, NFAT, and NFκB were then assessed by reporter assays. Briefly, the cells were transfected with reporter vectors and stimulated for 5.5 h with 50 µg/ml PHA and 200 ng/ml PMA in media containing 0, 15 or 50 µM zinc acetate. Reporter gene activities were then measured. To define the impact of AP-1 activity on IFN-γ expression, cells with or without reporter genes were pre-treated with the c-Jun N-terminal kinase (JNK) inhibitor SP600125 at various concentrations for 1 h before stimulation, then activated to evaluate AP-1 activity or IFN-γ expression. Finally, the effect of exogenous zinc on AP-1 reporter activity was evaluated under various stimulating conditions to elucidate the role of exogenous zinc on the AP-1 pathway. The cells were stimulated with different PMA/PHA ratios, various concentrations, or PMA/ionomycin. The effect of zinc on AP-1 activity was also evaluated by pre-treating cells for 30 min with novel or conventional protein kinase C (PKC) inhibitors (rottlerin or Gö-6976, respectively).

RNA isolation and real-time quantitative polymerase chain reaction (PCR)

To quantify IFN-γ gene expression, real-time quantitative PCR (LightCycler®; Roche) was performed. All values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. Total RNA was isolated by ISOGEN and treated with DNase to avoid genomic DNA contamination in accordance with the manufacturer's instructions. RNA (2 µg) was reverse-transcribed by Superscript™ II, excluding those samples using SP600125. Reverse-transcription was performed in reaction mixtures containing 2.5 µM of oligo-deoxyribothymidine (oligo$(dT)_{20}$) primers, 0.5 mM deoxyribonucleotide triphosphates, 10 mM dithiothreitol, and 120 units Superscript™ II in 40 µl of first strand buffer. The cDNA was precipitated, washed in ethanol, dissolved in Tris-EDTA buffer, and stored at −80 °C until quantification. Samples treated with SP600125 were reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit following the manufacturer's protocol. Concentrations of cDNA for IFN-γ and GAPDH were measured by quantitative PCR using LightCycler® FastStart DNA Master ^{PLUS} SYBR Green I. The sequences of the specific primers and annealing temperatures were as follows: human IFN-γ (GenBank accession number BC070256) sense primer 5′-TGGGTTCTCTTGGCTGTTACT-3′ and anti-sense primer 5′-GTATTGCTTTGCGTTGGACAT-3′, 59 °C; human GAPDH (GenBank accession number NM_002046) sense primer 5′-GATGACATCAAGAAGGTGGTG-3′ and anti-sense primer 5′-GCTGTAGC-CAAATTCGTTGTC-3′, 57 °C.

Measurement of cell viability

Cells were seeded in a 96-well plate at a density of 4×10^5 cells/ml and treated with the indicated conditions for 5 h. The cells were then incubated with 0.5 mg/ml MTT for an additional 3 h. Formazan crystals in the cells were collected and resolved in 40 mM hydrochloride/2-propanol to measure optical absorbance at 570 nm by spectrophotometry.

Reporter assay

pAP1-Luc and pNFκB-SEAP or pNFAT-SEAP were co-transfected into Jurkat cells with pCMVβ by lipofectamine™2000 according to the manufacturer's protocol. After an 18 h incubation in media containing 10% FBS, the cells were stimulated in serum-free medium supplemented with 0.5% AlbuMAX II. pTAL-Luc and pTAL-SEAP plasmids lacked any responsive element and were thus used as negative controls. The cells were harvested for luciferase and β-galactosidase assays at 5.5 h after stimulation. The supernatants were collected for SEAP (secreted form of human placental alkaline phosphatase) assay. The cells were rinsed with PBS, lysed in 30 µl of reporter lysis buffer, and cellular luciferase activity was measured using the Luciferase Assay System. As an internal control, β-galactosidase activity was determined by hydrolysis of chlorophenol red β-D-galactopyranoside at 570 nm by spectrophotometry. SEAP activity in the culture supernatant was analyzed by the Great EscAPe™ SEAP Chemiluminescence Fluorescence Detection Kit. Prior to assay analysis, the zinc concentration of each sample was adjusted to 45 µM by addition of zinc acetate to avoid zinc-dependent alteration of enzymatic activity.

Statistics

Statistical analysis of the data was performed using two-way ANOVA and/or the Tukey multiple comparisons test at a significance level of $P<0.05$. Comparisons between two groups were performed by the Student's *t*-test at a significance level of $P<0.05$.

Results

Altered endogenous expression of IFN-γ during T cell activation

To characterize endogenous IFN-γ expression during activation, Jurkat cells were stimulated with PHA and/or PMA. IFN-γ expression in the cells showed maximum induction during 4.5–8 h after various stimulations (data not shown). As shown in Fig. 1, cells treated with either PMA or PHA alone induced IFN-γ expression. In contrast, stimulation of cells with both 50 µg/ml of PHA and 200 ng/ml of PMA synergistically induced IFN-γ expression 11.2–11.8-fold greater than that of cells induced by either PMA or PHA alone. Cells without stimulation did not express IFN-γ.

Inhibition of endogenous IFN-γ mRNA by exogenous zinc

Zinc concentrations of 50 µM or greater were sufficient to attenuate IFN-γ expression (Fig. 2A) and significantly influenced IFN-γ expression ($P=0.017$); however, the time course of expression was similar between treatments (Fig. 2B). In cells without 50 µM of zinc treatment, the IFN-γ mRNA level was significantly elevated between 6 and 8 h when compared to that at 3 h after stimulation. However, a significant induction of IFN-γ expression in cells treated with zinc was detected

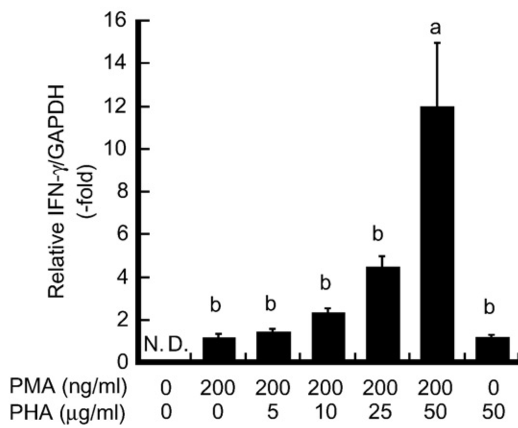

Fig. 1. Effects of various combinations of PHA and PMA on transcription of IFN-γ mRNA. Jurkat cells (1×10^6 cells/ml) were stimulated by PMA and/or PHA at the indicated concentration. The relative IFN-γ expression was compared to the average expression of cells treated with 200 ng/ml PMA without PHA. The values are shown as the mean±SEM in triplicate experiments. Values not sharing the same letter were significantly different as measured by the Tukey multiple comparison test ($P<0.05$).N.D. = not detected.

only at 8 h after stimulation. Viability of activated cells decreased approximately 30% when compared to naive cells (Fig. 2C). However, zinc did not reduce cell viability regardless of activation states.

Effects of zinc on inflammatory signaling

As shown in Fig. 3A, exogenous zinc reduced AP-1 reporter gene activity, and 50 μM of zinc significantly inhibited 17.6% of activity in zinc-free medium. Treatment with a JNK selective inhibitor (SP600125) greatly reduced AP-1 reporter gene activity (Fig. 3B). Thus IFN-γ expression was significantly diminished in an AP-1 activity-dependent manner (Fig. 3C). In these conditions, treatment of the activated cells with 10 μM or 50 μM of SP600125 suppressed AP-1 reporter gene activity by 13.2% or 43.4%, and IFN-γ expression by 50.6% or 96.5%, respectively. Although a zinc-depleted condition (0 μM) significantly reduced NFAT reporter gene activity compared to that in human serum containing normal zinc concentration (15 μM), addition of 50 μM of supplemental zinc did not alter the activities of NFAT and NFκB reporter genes (Fig. 3D and E).

Target pathway of the zinc-mediated down-regulation of AP-1 activity

In cells activated with the same ratio of PMA/PHA (200 ng/ml:50 μg/ml), a significant inhibitory effect of zinc on AP-1 reporter gene activity was observed under each condition (Fig. 4A). When cells were stimulated with increased PMA/PHA ratios, addition of zinc significantly suppressed AP-1 reporter gene activity in those cells. Alternatively, when cells were treated with zinc under lower PMA/PHA ratio conditions, addition of zinc produced neither a reducing or upregulating effect on AP-1 reporter gene activity. However, when calcium signaling was strongly induced with 4 μM of ionomycin, zinc was not able to downregulate AP-1 activity, even though AP-1 activity was reduced in cells treated with PMA±1 μM of ionomycin (Fig. 4A and B). Rottlerin, an inhibitor of calcium-independent PKC, eliminated the inhibitory effect of zinc on AP-1 activity, while the calcium-dependent PKC inhibitor, Gö-6976, did not eliminate this effect (Fig. 4C).

Discussion

Expression of IFN-γ in activated T lymphocytes is known to induce early inflammatory events in the intima (Shimizu et al., 2006). Thus control of IFN-γ expression is important for preventing progression of chronic inflammatory diseases such as atherosclerosis. This study is the first to demonstrate that zinc inhibits IFN-γ expression via suppression of the calcium-independent PKC–AP-1 pathway.

While normal human serum contains 12–18 μM of zinc (Cousins, 2006), clinically severe zinc deficiency and acute inflammation decrease serum zinc concentrations to 4–5 μM or less. We determined that the commonly used complete medium with 10% FBS contained 4.8 μM of

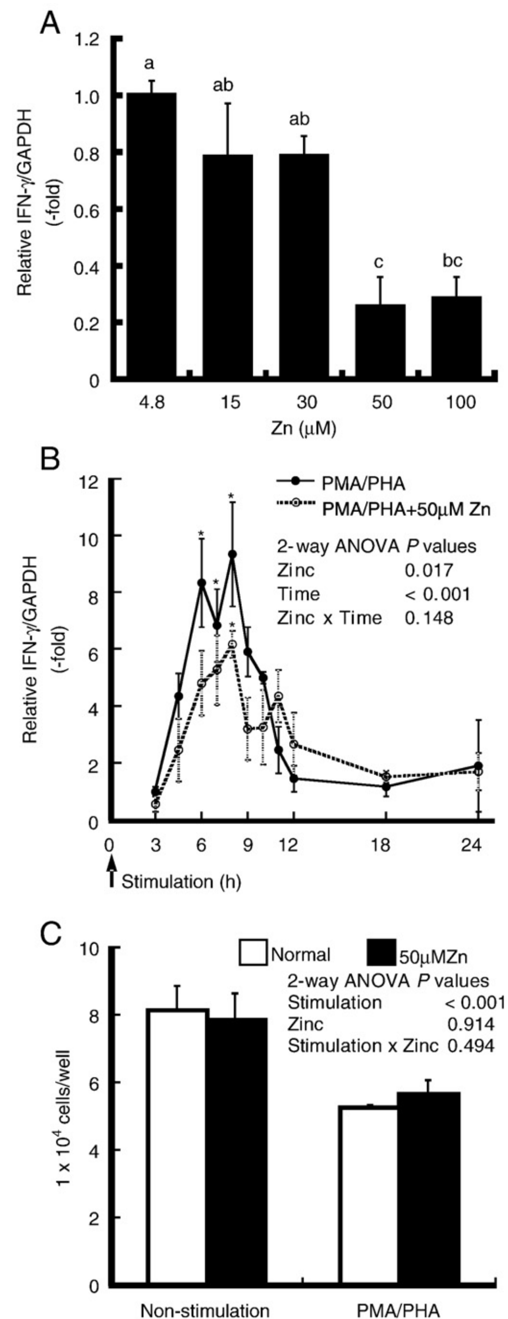

Fig. 2. Effects of zinc on IFN-γ transcription and cell viability. (A) Dosage effects of zinc on IFN-γ expression. Cells (2.25×10^6 cells/ml) were stimulated with various concentrations of zinc, and the IFN-γ transcription level for each sample was calculated to the relative amount of 'without addition' group. The values not sharing a common letter were significantly different by the Tukey multiple comparison test ($P<0.05$). (B) Changes in IFN-γ expression in activated cells with or without exogenous zinc. Cells were stimulated with (open circle) or without (closed circle) 50 μM of zinc. The IFN-γ transcription level of each sample was calculated to the relative amount of transcription in the control group at 3 h. Asterisk denotes a significant difference from the value at 3 h by the Tukey multiple comparison test ($P<0.05$). *P* values were indicated for each parameter estimated by two-way ANOVA. (C) Cell viability during the period of accelerated IFN-γ expression. All values are shown as the mean±SEM in triplicate experiments.

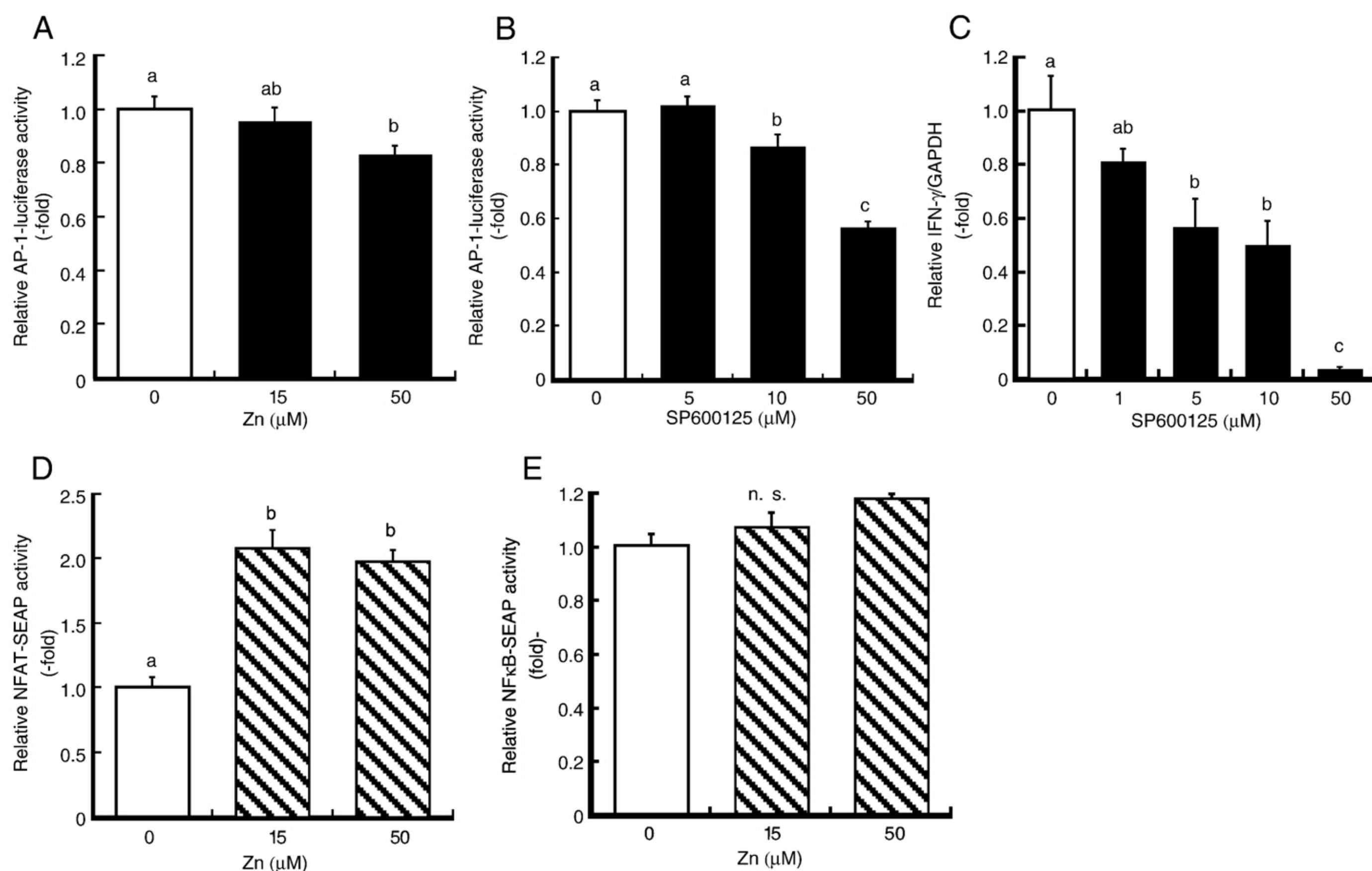

Fig. 3. Effects of zinc on the AP-1 signaling pathway and a role for AP-1 activity in IFN-γ expression. The values are indicated as the mean ± SEM (n = 5). Values not sharing the same letters were significantly different as measured by the Tukey multiple comparison test (P < 0.05). n.s. = not significant. (A) Activities of the AP-1 luciferase reporter vector in the activated cells were measured. Effects of the JNK inhibitor SP600125 on (B) AP-1 luciferase reporter activity and (C) IFN-γ expression were measured. Activities of (D) NFAT-SEAP and (E) NFκB-SEAP reporter vector in the activated cells were also measured.

zinc. Therefore, 10% FBS medium is considered to be zinc-deficient, and the zinc-free medium used here (0 μM) is considered to be severely zinc-deficient. We examined inflammation-related signaling under the three zinc concentrations (0, 15, and 50 μM), each reflecting severely deficient, normal, and fortified zinc conditions, respectively. Under these concentrations, AP-1 reporter gene activity seemed to decrease with an increase in zinc concentration (Fig. 3A). The zinc-mediated suppression of AP-1 and/or NFκB signaling was reported in many other cell types (Oteiza et al., 2000; Schott-Ohly et al., 2004; Mackenzie et al., 2006). However, there have been no reports on suppression of Th1-type inflammation by zinc mediated through the AP-1 signaling pathway in human T lymphocytes. In the present study, we attempted to clarify this mechanism. SP600125 is a commonly available JNK-1 and -2 selective inhibitor that blocks c-Jun phosphorylation, resulting in impairment of AP-1 activity (Park et al., 2004; Kwakkel et al., 2007; Leventaki et al., 2007). Treatment with 10 μM of SP600125 decreased the expression of IFN-γ, though partially inhibiting AP-1 activity as well (Fig. 3B and C). The reported IC_{50} values were 0.04 μM for JNK-1 and -2 in an enzyme-based assay, 5–10 μM for c-Jun phosphorylation in a cell-based assay, and 7 μM for IFN-γ expression induced by PMA/PHA in Jurkat cells (Bennett et al., 2001). Treatment with less than 50 μM of SP600125 completely inhibited c-Jun phosphorylation in activated Jurkat cells, regardless of results from the AP-1 reporter assay (Fig. 3B). Since AP-1 is a complex containing c-Jun, c-Fos and other transcription factors in various ratios, dynamics of genuine AP-1 activity in zinc-treated cells may be more greatly modulated than expected from the AP-1-luciferase results alone. In addition, AP-1 responsive elements in the genome often co-exist and cooperate with binding sites of another transcription factors including NFAT and NFκB. However, AP-1 responsive elements on the reporter vector did not contain these sites. Therefore, possibly even a partial decrease of AP-1 luciferase activity by 50 μM of zinc may be accompanied by a substantial reduction of AP-1 activity and IFN-γ expression.

Under the zinc-depleted condition (0 μM), NFAT activity was remarkably downregulated compared to that in the physiologically normal concentration (15 μM). In contrast, there was no significant effect on NFAT activity when the normal zinc concentration was increased to 50 μM. The detailed mechanisms underlying these molecular interactions are unclear. However, expression of NFATc1, a major NFAT isoform, may be favored by zinc due to a metal response element rich region near the open reading frame. Metal response element binding transcription factor-1 has a Cys2His2 zinc finger DNA-binding domain important for zinc-dependent activation of gene expression (Laity and Andrews, 2007). Therefore, zinc may have important roles not only as a negative regulator of AP-1 activity, but also as an essential factor for basal NFAT activity. Impairment of NFAT activity under the zinc-depleted condition may explain clinical immunodeficiency caused by lack of zinc. Results from reporter assays in this study clearly demonstrated that the effects of zinc on AP-1 and NFAT signaling were independent of one another (Fig. 3A and D), providing further support for the clinical application of zinc. Clinical zinc treatment may support Th1 function through NFAT activity without excessive activation accompanied by suppression of AP-1-related inflammation.

T cell activation was synergistically controlled by different pathways, diacylglycerol and calcium signaling stimulated by PMA and PHA/ionomycin, respectively (Fig. 1). Interestingly, the inhibitory effect of zinc on AP-1 activity depended on the PMA/PHA or PMA/ionomycin ratio (Fig. 4A and B). Moreover, inhibition was not diminished in cells treated with a conventional PKC (PKC-α/β)

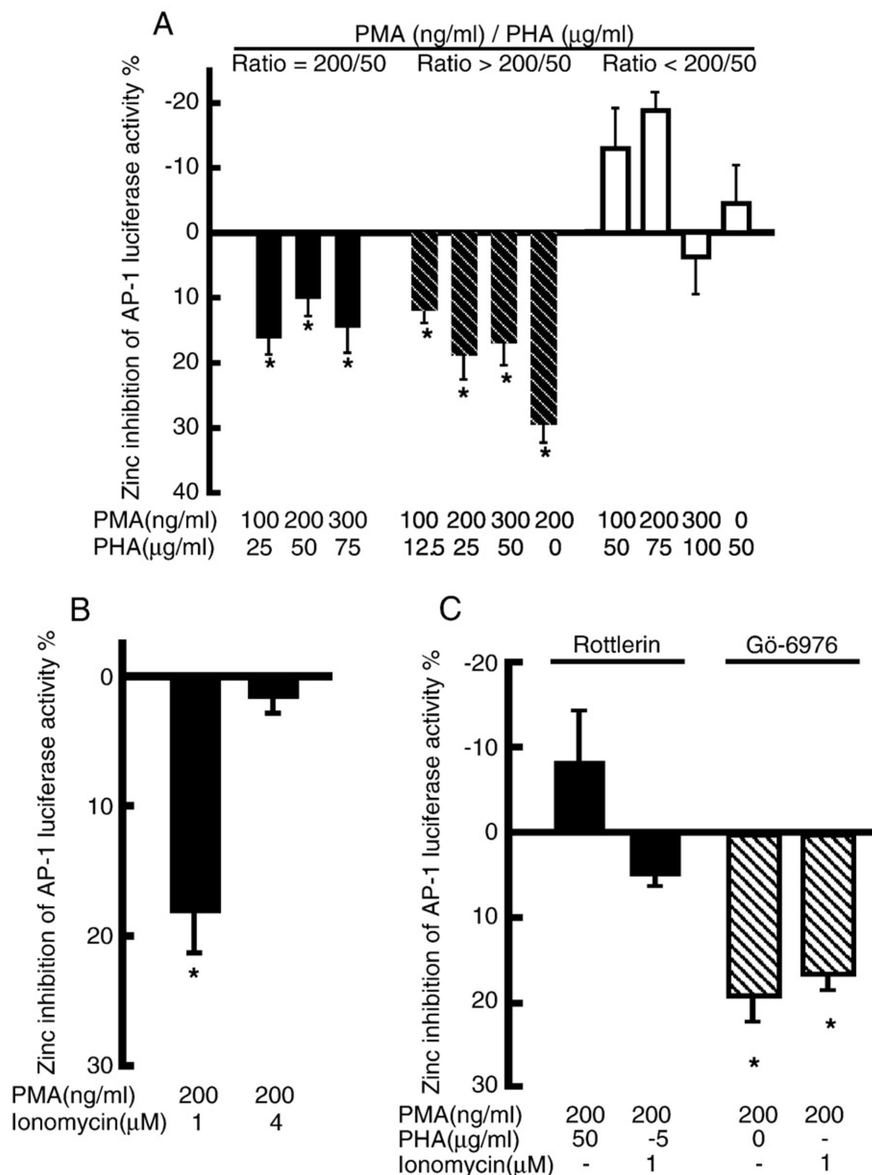

Fig. 4. Inhibitory effects of zinc on the AP-1 signaling pathway under various stimulatory conditions. Stimulating conditions were as indicated. Data are shown as the mean ± SEM of inhibition rates of AP-1 luciferase activities in cells treated with exogenous zinc (50 µM) compared to those of cells in the zinc-free medium ($n = 5$). The asterisk denotes that AP-1 activity in the zinc-treated cells was significantly lower than that of cells activated in the zinc-free medium ($P < 0.05$). (A) Cells were activated with various combinations of PMA/PHA. (B) Cells were stimulated with PMA 200 ng/ml plus ionomycin (1 or 4 µM) instead of PHA. (C) Cells were activated under the presence of the calcium-independent PKC-δ/θ inhibitor, rottlerin (10 µM; black bars), or the calcium-dependent PKC-α/β inhibitor, Gö-6976 (50 nM; slashed bars).

selective inhibitor, but was abolished in those cells treated with rottlerin, a selective inhibitor of the calcium-independent PKC-θ in T lymphocytes (Fig. 4C). Therefore, zinc may have the potential to control the PKC-θ-related signaling pathway, which is essential for mature T cell activation (Bi et al., 2001; Isakov and Altman, 2002; Springael et al., 2007).

Calcium-independent PKC activation upregulates AP-1 through the MKK4 (mitogen-activated protein kinase kinase 4)–JNK–c-Jun–AP-1 pathway and Ras–ERK (extracellular signal-regulated kinase)–c-Fos–AP-1 pathway (Isakov and Altman, 2002). Although the mechanism of AP-1 suppression by zinc has not been elucidated, the putative pathway seems to be the T cell-specific PKC-θ–MKK4–JNK–AP-1 pathway. PKC-θ is known as a dominant regulator for MKK4, an activator for the mitogen-activated protein kinase (MAPK) cascade during T cell activation (Isakov and Altman, 2002). In this study, zinc inhibition of AP-1 was more evident when cells were stimulated with an increased PMA/PHA or PMA/ionomycin ratio (Fig. 4A and B). Although PKC-θ is calcium-independent, the downstream MKK4 is synergistically enhanced by calcium-dependent calcineurin (Isakov and Altman, 2002). In this study, enhanced calcium signaling abrogated the inhibitory effect of zinc on AP-1 (Fig. 4B). It is possible that increased calcium signaling in zinc-treated cells may remove the suppression of MKK4 activity through calcineurin. The Ras–ERK–AP-1 pathway is also upregulated by both calcium-independent and -dependent PKCs (Isakov and Altman, 2002). This pathway, however, is unlikely to be a target of zinc considering that the conventional PKC–AP-1 pathway was not suppressed by zinc in rottlerin-treated cells. Therefore, zinc may inhibit the primary signal derived from PKC-θ before being activated by calcineurin.

Another possible zinc target may be the MAPK cascade. Recently, various zinc finger proteins were identified that downregulated AP-1 signaling through the MAPK pathway (Huang et al., 2004, 2006; Liu et al., 2004; Ou et al., 2005; Cao et al., 2005; Yang et al., 2005; Zhao et al., 2006). Activities of the zinc finger proteins may be strictly controlled by labile zinc as these proteins contain 3–18 zinc fingers. We also confirmed that 50 µM of exogenous zinc acetate quickly elevated intracellular labile zinc within the nontoxic range (data not shown). Thus, zinc may regulate the T cell-

specific pathway of PKC-θ–MKK4 as well as downstream MAPK–JNK–AP-1 signaling. It is possible that activities of other transcription factors controlled by MAPK are also reduced. Attenuation of IFN-γ expression may be synergistically modulated by AP-1 and other transcription factors.

In the present study, 50 μM of zinc could significantly reduce IFN-γ expression without the presence of typical ionophores such as pyrithione (Fig. 2A and B). The concentration of zinc was not toxic to Jurkat cells (Fig. 2C) (Sutherland et al., 2000). Pharmacological therapies administer high doses of zinc acetate (75–150 mg zinc per day) as a clinical drug called Galzin or Wilzin, for the amelioration of Wilson's disease with only mild side effects. It has been reported that serum zinc concentration was elevated approximately two-fold after an oral clinical dose of 50 mg zinc (Henderson et al., 1995). Although the zinc concentration used in the present study (50 μM) was approximately three-times higher than the normal serum concentration, it was far less than those used in other *in vitro* studies (>100 μM), which displayed anti-inflammatory effects of zinc (Ganju and Eastman, 2003; Tanaka et al., 2005). Thus even at low concentrations, zinc may have therapeutic potential for the regulation of inflammatory diseases. Prevention of zinc deficiency is important for avoiding further development of inflammatory diseases, including atherosclerosis.

In conclusion, exogenous zinc significantly suppressed IFN-γ expression by down-regulation of the calcium-independent PKC–AP-1 signaling pathway in activated Jurkat T cells.

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