Polaprezinc Down-Regulates Proinflammatory Cytokine-Induced Nuclear Factor-κB Activation and Interleukin-8 Expression in Gastric Epithelial Cells

TADAHITO SHIMADA, NAOMI WATANABE, YUKIO OHTSUKA, MOTORO ENDOH, KAZUO KOJIMA, HIDEYUKI HIRAISHI, and AKIRA TERANO

Second Department of Internal Medicine, Dokkyo University School of Medicine, Mibu, Tochigi, Japan

Accepted for publication June 10, 1999

ABSTRACT

Gastric epithelial chemokine response is a primary factor in the induction of gastric inflammation associated with Helicobacter pylori infection. Because sustained inflammation is a risk for gastric mucosal damage, agents that down-regulate inflammatory responses may be of therapeutic significance. We examined the effect of polaprezinc, a potent antiulcer agent, on proinflammatory cytokine-induced interleukin (IL)-8 expression in gastric epithelial cells. Because IL-8 expression is regulated by the transcription factor nuclear factor-κB (NF-κB), we also examined the effect of polaprezinc on NF-κB activity. MKN28 cells were used as a model of gastric epithelial cells. Secreted IL-8 was quantified by IL-8 specific enzyme-linked immunosorbent assay, and IL-8 mRNA expression was examined by Northern blot analysis. NF-κB activity was analyzed by electrophoretic mobility shift assay. Western blot analysis with anti-phospho-IκBα antibody was performed to assess IκBα phosphorylation. Polaprezinc-suppressed IL-8 secretion induced by tumor necrosis factor α (TNF-α) or IL-1β in a dose-dependent manner. IL-8 mRNA expression also was inhibited by polaprezinc. NF-κB activation in response to TNF-α, IL-1β, phorbol ester, and H2O2 was down-regulated by polaprezinc. Western blot analysis showed inhibition of TNF-α-induced IκBα phosphorylation in the presence of polaprezinc. Collectively, these results suggest that polaprezinc is a novel type of anti-inflammatory agent that down-regulates inflammatory responses of gastric mucosal cells.

The compound N-(3-aminopropionyl)-L-histidinato zinc (polaprezinc), a chelate of zinc and L-carnosine, is an antiulcer agent developed in Japan (Fig. 1) (Ueki et al., 1989). It is known that carnosine increases granulation tissue (Vizioli and Almeida, 1978; Nagai et al., 1986) and accelerates gastric ulcer healing in rats (Yamakawa, 1975). Zinc has been reported to have protective action against various experimental gastric lesions (Cho et al., 1976; Cho and Ogle, 1977, 1978), and clinical studies have shown antiulcer action of zinc in humans (Frommer, 1985; Alcala-Santaella et al., 1985; Varas-Lorenzo, 1986). Polaprezinc was originally designed to combine the beneficial effects of zinc and carnosine. Several reports have shown protective action of polaprezinc against experimental gastric lesions induced by various noxious agents (Ueki et al., 1989; Arakawa et al., 1990; Cho et al., 1991; Yoshikawa et al., 1991b; Hiraishi et al., 1999) and that polaprezinc accelerates gastric ulcer healing in humans (Morise et al., 1992). Although the mechanisms of antiulcer action of polaprezinc could be partly explained by its stimulatory effect on mucus secretion, membrane-stabilizing effect, and antioxidant properties (Arakawa et al., 1990; Cho et al., 1991; Yoshikawa et al., 1991a), they are not fully understood.

A growing amount of evidence shows that epithelial cells are playing an important role in the gastric mucosal cytokine network (Crabtree, 1996; Shimada and Terano, 1998). In Helicobacter pylori infection, gastric epithelial cells secrete large amounts of interleukin (IL)-8, a prototype of the CXC chemokines that recruit and activate neutrophils (Harada et al., 1996), in response to proinflammatory cytokines produced locally or in response to attachment of H. pylori to the cell surface (Crabtree et al., 1994). This epithelial chemokine response appears to be a primary factor in the induction of gastric inflammation in response to H. pylori infection. IL-8 mRNA expression is regulated by the transcription factor nuclear factor-κB (NF-κB) in combination with NF-IL-6 or activating protein-1 (AP-1) (Mukaida et al., 1990; Yasumoto, 1991). This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture in Japan. Part of this work was presented at the annual meeting of the American Gastroenterological Association in New Orleans, Louisiana, May 1998, and was published in abstract form (Gastroenterology 114:A1084, 1998).

ABBREVIATIONS: IL, interleukin; NF-κB, nuclear factor-κB, AP-1, activating protein 1; TNF-α, tumor necrosis factor α; PMA, phorbol-12-myristate-13 acetate; SSC, standard saline citrate; DTT, dithiothreitol; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; TBST, Tris-buffered saline/Tween 20; EMSA, electrophoretic mobility shift assay.
purification kit (Quiagen, Hilden, Germany), and sequence identity was confirmed by direct sequencing. Hybridization with human glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech, Palo Alto, CA) also was performed for standardization.

The filters were preincubated in 50% formamide, 5× sodium chloride–sodium citrate (SSC) solution, 5× Denhardt’s solution, 50 mM sodium phosphate (pH 6.8), 0.1% SDS, 5 mM EDTA, and 0.1 mg/ml denatured salmon sperm DNA for 16 h at 42°C. Heat-denatured cDNA probe (25 ng) was labeled with [γ-32P]dCTP (Amersham Pharmacia Biotech) with a random primer labeling kit (Rediprime kit; Amersham Pharmacia Biotech). Labeled cDNA probe was added to the solution, and hybridization was continued for 16 h at 42°C. Filters were washed with 2× SSC and 0.1% SDS for 20 min at room temperature, next with 2× SSC and 0.1% SDS for 30 min at 50°C, followed by washes with 0.1× SSC and 0.1% SDS for 15 min twice at 50°C. Autoradiography and densitometric analyses were performed with a BAS2000 Bioimaging System (Fujiy, Tokyo, Japan).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from −5 × 105 cells as described by Schreiber et al. (1989) with some modifications. After incubation with test agents, MKN28 cells were harvested and washed twice with ice-cold PBS. Cells were lysed in 800 μl of hypotonic buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride] on ice for 15 min, after which 50 μl of 10% Nonidet P-40 solution was added, and the mixture was vortexed vigorously for 15 s and centrifuged for 30 s at 12,000 rpm. The pelleted nuclei were resuspended in 50 μl of buffer B [50 mM HEPES (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol] and incubated on ice for 20 min with intermittent mixing. The tubes were centrifuged for 5 min at 12,000 rpm, and the supernatant containing nuclear extracts was collected. The protein concentration was determined with a Bio-Rad protein concentration assay kit (Bio-Rad Laboratories, Richmond, CA). The nuclear extracts were stored at −70°C until use.

A 25-mer NF-κB consensus oligonucleotide (5′-AGTTGAGGGAGCTTTCCAGCCGAG-3′) (Promega Biotec, Madison, WI) was end-labeled with [γ-32P]ATP (Amersham Pharmacia Biotech) with T4 polynucleotide kinase (Promega Biotec). Five micrograms of nuclear extract proteins was preincubated in 9 μl of a binding solution [4% (v/v) glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)] for 10 min at room temperature. After addition of the 32P-labeled oligonucleotide probe, the incubation was continued for 20 min, and DNA-protein complex formed was separated on a 5% nondenaturing polyacrylamide gel electrophoresis. The gel was then dried and analyzed with a BAS2000 Bioimaging System (Fujiy). An AP-1 consensus oligonucleotide probe (5′-GCCCTGATGATCCGAGCAAAAG-3′) (Promega Biotec) also was used in some experiments. In the supershift experiments, nuclear extracts were preincubated in the binding buffer with one of the antibodies against NF-κB subunits (p50, p52, p65, or c-Rel) (Santa Cruz Biotechnologies, Santa Cruz, CA) for 45 min at room temperature before the addition of the labeled probe.

Western Blot Analysis. MKN28 cells grown in 10-cm culture dishes were treated with test agents and directly collected in 150 μl of SDS-sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue. The cell lysates were sonicated for 15 s to shear high-molecular-weight DNA and reduce sample viscosity. After 10% SDS-polyacrylamide gel electrophoresis, the separated proteins were transferred onto a polyvinylidene difluoride membrane (Clear blot membrane-P, ATTO, Tokyo, Japan). Polyvinylidene difluoride membranes were blocked in Tris-buffered saline/Tween 20 (TBST) [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20] containing 5% nonfat dry milk (Bio-Rad Laboratories) overnight at 4°C. The membranes were then incubated with rabbit polyclonal antiphospho-IκBα (Ser32) antibody (New England BioLabs, Beverly, MA) or rabbit polyclonal anti-IκBα antibody (New England BioLabs) in TBST containing 5%
BSA overnight at 4°C. After washing with TBST three times for 5 min, the membranes were incubated with peroxidase-conjugated anti-rabbit IgG antibody (New England BioLabs) in TBST containing 5% nonfat dry milk for 1 h at room temperature. The membranes were washed again, and the specific bands were visualized with LumiGLO chemiluminescent reagent (New England BioLabs).

**Statistics.** Data were expressed as the mean ± S.D. Statistical analysis was performed using Student’s t test for unpaired data, and p values <.05 were considered to be significant.

**Results**

**Effect of Polaprezinc on Proinflammatory Cytokine-Induced IL-8 Secretion.** At first, we examined the effect of polaprezinc on IL-8 secretion induced by TNF-α or IL-1β in MKN28 cells. In the experiments shown in Fig. 2A, cells were serum-deprived for 24 h and treated with 10 ng/ml TNF-α alone or with increasing concentrations of polaprezinc (10–1000 μM). Each concentration of polaprezinc was preincubated with the cells for 3 h before the addition of TNF-α, and TNF-α treatment was performed in the constant presence of polaprezinc. Cell-free supernatants were harvested at a 16-h time point, and the IL-8 concentration of each sample was measured with human IL-8 specific enzyme-linked immunosorbent assay. Incubation with 10 ng/ml TNF-α caused a significant increase in IL-8 secretion by MKN28 cells (an ∼14-fold increase). However, compared with the IL-8 levels measured in the presence of 10 ng/ml TNF-α alone, polaprezinc caused a dose-dependent reduction in IL-8 secretion by MKN28 cells. A significant suppression in the IL-8 levels was observed in the presence of as low as 10 μM polaprezinc. In this series of experiments, 10, 100, 300, and 1000 μM polaprezinc caused suppression of the IL-8 secretion induced by 10 ng/ml TNF-α by ∼57, 60, 96, and 96%, respectively.

Figure 2B shows the effect of 300 μM polaprezinc on increasing concentrations of TNF-α (0.1–100 ng/ml)-induced IL-8 secretion by MKN28 cells. Polaprezinc (300 μM) was preincubated with the cells for 3 h before the addition of each concentration of TNF-α, and TNF-α treatment was performed in the presence of polaprezinc. The culture supernatants were collected at 16-h time point after addition of TNF-α. As seen in Fig. 2B, TNF-α dose-dependently increased IL-8 secretion by MKN28 cells (an ∼22-fold increase at 100 ng/ml TNF-α), and 300 μM polaprezinc completely abolished the stimulative effects of TNF-α.

Similar experiments were performed to examine the effect of polaprezinc on IL-1β-induced IL-8 secretion. The experimental procedures were the same as those for the experiments with TNF-α. As seen in Fig. 3A, IL-1β more potently induced IL-8 secretion by MKN28 cells than TNF-α, and 10 ng/ml IL-1β caused an ∼25-fold increase in the IL-8 secretion. Although 10 μM polaprezinc did not suppress 10 ng/ml IL-1β-induced IL-8 secretion, 100 μM polaprezinc caused ∼56% suppression, and 300 μM polaprezinc almost completely abolished the IL-8 secretion. Figure 3B shows IL-8 secretion induced by increasing concentrations of IL-1β (0.1–100 ng/ml) in the presence and in the absence of polaprezinc (300 μM). This concentration of polaprezinc potently inhibited IL-8 secretion induced by all concentrations of IL-1β tested, and the suppression was >90%.

We examined the effect of polaprezinc on the cell viability to check the toxicity of this drug. MKN28 cells grown in 96-well culture dishes (1 × 10^4 cells/well) were incubated with different concentrations of polaprezinc (10–1000 μM) for 24 h, and cell viability was then assessed with the WST-1 assay. Although 1000 μM polaprezinc caused a decrease in the cell viability (49.1 ± 5.5%, n = 6), lower concentrations of polaprezinc had no significant effect (102.6 ± 7.0%, 102.8 ± 10.4%, 99.8 ± 4.5%, and 97.3 ± 6.6% with 10, 30, 100, and 300 μM polaprezinc, respectively, n = 6), suggesting that the effect described above of polaprezinc on IL-8 secretion was not due to cell toxicity.

**Effect of Polaprezinc on Proinflammatory Cytokine-Induced IL-8 mRNA Expression.** Northern blot analysis was performed to determine whether polaprezinc down-regulates TNF-α- or IL-1β-induced IL-8 mRNA expression in MKN28 cells. Cells were incubated with 10 ng/ml TNF-α or IL-1β in the presence and in the absence of increasing concentrations of polaprezinc. Polaprezinc was preincubated for 3 h before the addition of TNF-α or IL-1β, and the treatment of these proinflammatory cytokines was performed in the presence of polaprezinc. At a 4-h time point, culture supernatants were aspirated, and the cell monolayers were...
scraped. Total RNA was extracted, and standard Northern blot experiments were performed.

Figure 4 shows an experiment with TNF-α (a representative of three similar experiments). As seen in Fig. 4A, IL-8 mRNA expression was significantly up-regulated by TNF-α (10 ng/ml) and polaprezinc dose-dependently suppressed it. A densitometric analysis of this experiment (Fig. 4B) shows that the IL-8 mRNA:glyceraldehyde-3-phosphate dehydrogenase mRNA ratio was suppressed by 90% in the presence of 300 μM polaprezinc compared to the incubation with 10 ng/ml TNF-α alone. In the experiment shown in Fig. 5, MKN28 cells were stimulated with 10 ng/ml IL-1β (a representative of three similar experiments). IL-8 mRNA expression was significantly up-regulated by IL-1β, and it also was suppressed by polaprezinc in a dose-dependent manner (Fig. 5A). A densitometric analysis of this experiment (Fig. 5B) shows almost complete suppression of IL-8 mRNA expression by 300 μM polaprezinc. Thus, these results indicate that the inhibitory effects of polaprezinc on TNF-α- or IL-1β-induced IL-8 secretion are associated with the suppression of IL-8 mRNA expression.

**Effect of Polaprezinc on Proinflammatory Cytokine-Induced IL-8 NF-κB Activation.** Because IL-8 expression is mainly regulated by the transcription factor NF-κB (Mukaida et al., 1990; Yasumoto et al., 1992; Matsusaka et al., 1993; Aihara et al., 1997), EMSA experiments were performed to examine the effect of polaprezinc on the activation of NF-κB induced by proinflammatory cytokines. Incubation of MKN28 cells with TNF-α (10 ng/ml) for 90 min caused significant up-regulation of NF-κB-specific DNA-binding activity (Fig. 6). The retarded band was shifted to higher molecular weight when the nuclear extracts were preincubated with antibodies to the NF-κB subunits p50 or p65, whereas preincubation with antibodies to the NF-κB subunits p52 or c-Rel had no effect, indicating that NF-κB in MKN28 cells mainly exists as a heterodimer of p50 and p65. The results of the supershift assay are confirmation that the observed bands were indeed NF-κB. The disappearance of the retarded band in the presence of a 100-fold excess amount of unlabeled NF-κB consensus oligonucleotide reconfirms that the band was NF-κB.

Figure 7 shows effects of different concentrations of polaprezinc (10–1000 μM) on TNF-α (10 ng/ml)-induced NF-κB activation. MKN28 cells were preincubated with each concentration of polaprezinc for 3 h, and then incubated with TNF-α for 90 min in the presence of polaprezinc.
Fig. 7, polaprezinc inhibited NF-κB activation induced by TNF-α in a dose-dependent manner, and 300 μM polaprezinc completely abolished its activation. IL-1β (10 ng/ml)-induced NF-κB activation was similarly inhibited by polaprezinc (data not shown). Because intracellular signal transduction pathways for TNF-α and IL-1β differ, these results suggest that polaprezinc acts at a step where signaling pathways of both cytokines converge with regard to NF-κB activation. We also examined the effect of polaprezinc on the activation of NF-κB induced by PMA (0.1 μM) (3-h incubation) and H₂O₂ (1 mM) (1-h incubation). Figure 8 shows that both PMA and H₂O₂ induced significant NF-κB activation in MKN28 cells and that polaprezinc (300 μM) completely blocked it.

Because polaprezinc is a chelate compound of zinc and L-carnosine, we next compared the effect of zinc with that of L-carnosine on TNF-α-induced NF-κB activation in MKN28 cells. Cells were preincubated with ZnSO₄ (100 or 300 μM) or L-carnosine (100 or 300 μM) for 3 h and then incubated with TNF-α (10 ng/ml) for 90 min in the presence of ZnSO₄ or L-carnosine. As shown in Fig. 9, either concentration of ZnSO₄ completely blocked NF-κB activation induced by TNF-α, whereas L-carnosine had no significant effect, suggesting that the observed effects of polaprezinc are mediated by zinc. We also checked the effect of ZnSO₄ on IL-8 secretion. In this series of experiments, 10 and 100 μM ZnSO₄ caused suppression of the IL-8 secretion induced by 10 μM TNF-α (2.79 ± 0.96 ng/ml, n = 4) by ~64 and 98%, respectively.

Effect of Polaprezinc on IκB-α Phosphorylation Induced by TNF-α. In the cytosol, NF-κB is inactive when complexed with IκB proteins (Baeuerle and Baltimore, 1996). Phosphorylation of IκB-α at Ser32 and 36 triggers dissociation of IκB from NF-κB complex, which results in NF-κB activation and translocation into the cell nucleus (Brown et
Dissociated IκB proteins are subjected to proteasome-mediated degradation (Brown et al., 1995; Traenckner et al., 1995). We examined the effect of polaprezinc on TNF-α (10 ng/ml)-induced IκB-phosphorylation (Ser32) in MKN28 cells. Cells were preincubated with 300 μM polaprezinc for 3 h and then treated with TNF-α (10 ng/ml) for 5, 10, 15, and 30 min in the constant presence of 300 μM polaprezinc. Control cells were similarly treated with TNF-α in the absence of polaprezinc. Western blot analysis was performed with anti-phospho-IκB-α (Ser32) antibody and anti-IκB-α antibody.

As shown in Fig. 10A, TNF-α transiently increased the amount of phosphorylated IκB-α in the absence of polaprezinc. An experiment with anti-IκB-α antibody showed a gradual decrease in the total amount of IκB-α protein on TNF-α treatment, suggesting the degradation of IκB-α after phosphorylation. However, in the presence of 300 μM polaprezinc (Fig. 10B), TNF-α treatment had no significant effect on the level of phosphorylated IκB-α. Total amount of IκB-α was constant during a 30-min incubation with TNF-α in the presence of polaprezinc. These results suggest that polaprezinc suppresses IκB-α phosphorylation and subsequent degradation in response to TNF-α treatment in MKN28 cells.

**Effect of Polaprezinc on AP-1 Activity.** Because AP-1 also may affect IL-8 expression (Yasumoto et al., 1992), we examined the effect of polaprezinc on AP-1-specific DNA-binding activity in MKN28 cells. As shown in Fig. 11, AP-1 activity was present in control cells, and it was slightly up-regulated by incubation with 10 ng/ml TNF-α, 10 ng/ml IL-1β, or 1 mM H₂O₂, whereas PMA had a more potent effect. Preincubation of the cells with 300 μM polaprezinc for 3 h completely abolished AP-1 activity, indicating that polaprezinc also down-regulates AP-1 activity in addition to NF-κB.

**Discussion**

Polaprezinc is a chelate compound of zinc and t-carnosine (Ueki et al., 1989), and previous studies showed its useful-
NF-kB to its cognate DNA site also is reported to be redox (reduction-oxidation)-sensitive (Sen and Packer, 1996). Various kinds of antioxidants, such as N-acetylcysteine, dimethyl sulfoxide, and α-lipoic acid, have been reported to down-regulate NF-kB activation in a wide range of cell types (Sen and Packer, 1996). Our previous study also demonstrated that NF-kB activation and IL-8 expression are redox-sensitive in MKN28 cells (Shimada et al., 1999). Because polaprezinc has been reported to have antioxidant properties (Yoshikawa et al., 1991b), the inhibitory effect of polaprezinc on NF-kB activation and IL-8 expression observed in the present study may be explained by its antioxidant properties. Because polaprezinc suppressed IκB-α phosphorylation induced by TNF-α treatment (Fig. 10), the primary site of polaprezinc’s action should be IκB kinase or upstream of IκB kinase. If polaprezinc’s action can be ascribed to its antioxidant properties, there may be redox-sensitive sites around IκB kinase in the signaling pathway leading to NF-kB activation. However, we cannot exclude the possibility that polaprezinc acts at multiple sites inside the cells.

We compared the effects of zinc and l-carnosine, components of polaprezinc, on NF-kB activation (Fig. 9). ZnSO4 (100–300 μM) had a potent inhibitory effect on TNF-α-induced NF-kB activation, whereas l-carnosine (100–300 μM) had no significant effect. Thus, the observed inhibitory effect of polaprezinc on NF-kB activation is likely to be mediated by its component, zinc. Zinc is known to have a variety of biological effects, including antioxidant properties (Yoshikawa et al., 1991a). Connell et al. (1997) showed that, in endothelial cells, zinc protects against cytokine-mediated activation of NF-kB and AP-1, up-regulation of inflammatory cytokines, and endothelial dysfunction. Consistent with this study, we found that polaprezinc inhibits AP-1-specific DNA-binding activity in MKN28 cells (Fig. 11). AP-1 also is known to be involved in the expression of genes relating to inflammatory responses (Connell et al., 1997). It should be noted that AP-1 is another redox-sensitive transcription factor (Sen and Packer, 1996) and certain antioxidant compounds are capable of influencing AP-1 activity (Sen and Packer, 1996). Additional studies are needed to determine whether polaprezinc’s inhibitory effect on AP-1 activity can be ascribed to its antioxidant properties. Yasumoto et al. (1992) reported that IL-8 expression is affected by AP-1 in addition to NF-kB in certain cell types. Although we have not determined the relative importance of these transcription factors in terms of IL-8 expression in MKN28 cells, it is possible that the potent inhibitory effect of polaprezinc on IL-8 expression in this cell line is partly due to the inhibition of AP-1 activity.

When administered p.o., polaprezinc adheres to the surface of the gastric mucosal layers, and its adhesiveness to gastric mucosa is superior to that of ZnSO4 or ZnSO4 + l-carnosine (Seiki et al., 1992). Therefore, although NF-kB is ubiquitously expressed in most tissues, polaprezinc may be useful as an agent to specifically down-regulate NF-kB activation in the gastric mucosa and to normalize the dysregulation of gastric mucosal cells under prolonged inflammatory conditions, such as H. pylori-associated gastritis. This study may provide a theoretical basis for the use of this agent as a novel type of anti-inflammatory drug to control gastric inflammatory responses.
References


