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Preliminary report

Prodigiosin isolated from *Hahella chejuensis* suppresses lipopolysaccharide-induced NO production by inhibiting p38 MAPK, JNK and NF-κB activation in murine peritoneal macrophages

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Abstract

Prodigiosin was isolated from marine bacteria *Hahella chejuensis* which has been recently discovered from Marado, Cheju Island, Republic of Korea. Immunosuppressive properties have been reported for prodigiosin members such as undecylprodigiosin, metacycloprodigiosin, prodigiosin and its synthetic analogue PNU156804 (PNU). However, the effect of this agent on macrophage function has not been characterized in detail. In the present study, we examined the effects of prodigiosin on the production of inflammatory cytokines and nitric oxide (NO) in lipopolysaccharide (LPS)-activated murine macrophage. When thioglycollate-elicited macrophages pre-exposed to prodigiosin (1–100 ng/ml) were stimulated with LPS, pretreatment with prodigiosin resulted in the inhibition of NO production and inducible nitric oxide synthase (iNOS) protein and mRNA expression in a concentration-dependent manner. In contrast, the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 was not altered. Inhibition of iNOS protein expression appears to be at the transcriptional level, since prodigiosin decreased LPS-induced NF-κB activity through preventing the degradation of IkBα, with significant inhibition achieved following pretreatment with prodigiosin. However, prodigiosin did not exert any effect on AP-1 activity. Prodigiosin blocked phosphorylation of p38 mitogen-activated protein kinase (MAPK) and c-Jun NH_2-terminal kinase (JNK), but not that of extracellular signal-regulated kinase 1/2 (ERK 1/2). These results indicate that the inhibition of these signaling molecules expression was correlated with the reduced production of NO in macrophages. Taken together, the present data suggest that prodigiosin reduces NO production and iNOS expression by inhibiting LPS-triggered p38 MAPK and JNK phosphorylation and NF-κB activation, thereby implicating a mechanism by which prodigiosin may exert its immunosuppressive effects.

Keywords: *Hahella chejuensis*; Prodigiosin; Macrophages; Nitric oxide; NF-kB; MAPK

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1. Introduction

There has been increasing interest in the discovery and development of novel pharmaceuticals from marine microorganisms that have the same or better immunosuppressants accompanied by less side effects. Recently, the only species of the genus, *Hahella chejuensis*, was isolated from the coastal marine sediment of the southernmost island in Korea and the complete genome sequence of *H. chejuensis* in its class was determined [1]. This bacterium produces abundant extracellular polysaccharides and prodigosin [2]. Prodigiosins are a family of natural red pigments containing three pyrrole rings produced by *S. marcescens, Pseudomonas magnesiorubra, Vibrio psychroerythrus* and other bacteria. Some members of this family, including prodigisin, have been suggested to have immunosuppressive activity, which suppress cytotoxic T cells by inhibition of acidification of intracellular organelles without affecting B cell-mediated immune functions, different from that of the other well known immunosuppressors such as cyclosporine A, FK506 and rapamycin [3–5]. However, the effect of prodigiosin on macrophage function is less well understood.

Macrophages have been shown to be important components of the host defenses against bacterial infections and murine tumor cells (lymphoma and mastocytoma) [6,7]. Peritoneal macrophages can be stimulated by a variety of agents such as IFN-γ, lipopolysaccharides, or other microbial products [8–10] and some of these have also been shown to trigger the release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and nitric oxide (NO) and to induce tumoricidal activity by the macrophages [11–15]. However, overexpression of inflammatory mediators such as cytokines and NO has been implicated in the pathogenesis of many disease processes. The control of macrophage overproduction of these mediators should greatly facilitate the treatment of many immunoinflammatory diseases such as septic shock, rheumatoid arthritis, cerebral malaria, and autoimmune diabetes [16,17]. In macrophages, inflammatory mediators are regulated primarily at the level of mRNA expression via the involvement of transcription factors such as NF-κB. A large number of studies have shown that different signaling pathways participate in the activation of macrophages by various stimuli [18–21]. Previous studies have demonstrated that the NF-κB and mitogen-activated protein kinase (MAPK) pathway mediate the activation of human monocyte [22–24]. In addition, LPS is known to activate a series of MAP kinases, such as extracellular signal-regulated kinase 1/2 (Erk1/2), p38 mitogen-activated protein kinase and c-Jun NH_2-terminal kinase (JNK), in macrophages [25–28]. However, the cellular signaling and molecular mechanisms responsible for pharmacological activity by prodigiosin are not known in macrophages.

In the present study, we determine the effect of prodigiosin on in vitro production of inflammatory cytokines and NO in order to gain a further insight into the mechanisms whereby this compound may mediate its immunosuppressive action in vivo.

2. Materials and methods

2.1. Isolation of prodigiosin

Prodigiosin was extracted with a mixture of methanol/1N HCl (24:1, v/v) from the supernatant of *H. chejuensis* culture which was grown on Marine Broth (Difco) for 24–48 h at 300 °C with vigorous shaking, as previously described [1]. Red-colored fraction was purified through high-performance liquid chromatography (LC). Following LC using acetonitrile and water (with 0.1% formic acid) as the mobile phase at a flow rate of 0.2 ml/min, ESI-MS was carried out with a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with a Finnigan electrospray source. To determine the molecular structure, ^{1}H NMR (CD3OD, 300 MHz) and ^{13}C NMR (CD3OD, 75 MHz) analyses were performed, resulting in the raw data of 6.94 (m, 1H), 6.71 (m, 1H), 6.66 (s, 1H), 6.39 (s, 1H), 6.21 (m, 1H), 6.01 (s, 1H), 3.89 (s, 3H), 2.37 (t, 2H), 2.27 (s, 3H), 1.53 (m, 2H), 1.33 (m, 4H), 0.90 (t, 3H) (^{1}H NMR) and 169.6, 160.2, 141.0, 135.8, 129.9, 129.2, 125.1, 123.0, 120.6, 115.9, 113.1, 110.9, 95.9, 58.9, 32.7, 31.8, 26.6, 23.6, 14.4, 11.5 (^{13}C NMR).

2.2. Mice, chemicals and reagents

The C57BL/6 male mice (6–8 weeks old, 17–21 g) were obtained from Charles River Breeding Laboratories (Atsugi, Japan). Unless otherwise indicated, all the chemicals were purchased from Sigma Chemical Co. (St Louis, MO). The RPMI 1640 medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY). IL-1, IL-6 and TNF-α ELISA kits were purchased from R&D System (Minneapolis, MN). The antibodies against inducible NO synthase (iNOS), IκB-α, Erk1/2, phospho-Erk1/2, p38, phospho-p38, JNK, phospho-JNK and β-actin were obtained from Santa Cruz Biotechnology, USA. All tissue culture reagents, the thioglycollate broth and prodigiosin were assayed for any endotoxin contamination using the Limulus lysate test (E-Toxate kit, Sigma) and the level of endotoxin was found to be < 10 pg/ml.

2.3. Isolation of inflammatory peritoneal macrophages

The thioglycollate-elicited peritoneal-exudate cells were obtained from C57BL/6 male mice (6–8 weeks old, 17–21 g) after they were given an intraperitoneal injection of 1 ml Brewer. Thioglycollate broth (4.05 g/100 ml) (Difco Laboratories, Detroit, ML) followed by a lavage of the peritoneal cavity with 5 ml of medium 3–4 days later. The cells were washed twice and resuspended in RPMI-1640 (GIBCO, Grand Island, NY)

containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The macrophages were isolated from the peritoneal exudate cells using the method described by Um et al. [20]. The peritoneal exudate cells were seeded on teflon-coated petri dishes (100×15 mm) at densities of $5–6\times10^5$ cells/cm_2 and the macrophages were allowed to adhere for 2–3 h at 37 °C in a 5% CO_2 humidified atmosphere. Teflon-coated petri dishes were prepared by spraying them with aerosolized teflon (Fisher Scientific, Pittsburgh, PA), which was followed by sterilization using ultraviolet light for 3 h. The non-adherent cells were removed by washing the dishes twice with a 10 ml prewarmed medium and incubating the dishes for 10 min at 4 °C. The supernatants were then carefully removed and discarded, and the plates were washed once with a prewarmed Dulbecco's Phosphate Buffered Saline solution (PBS) (GIBCO). Cold PBS (15 ml) containing 1.5% FBS was then added, which was followed by the addition of 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages were removed by rinsing the plates 10 times using a 10 ml syringe. The viability of the detached cells was assessed by trypan blue exclusion and the proportion of macrophages was determined after cytoplasmic staining with acridine orange using fluorescence microscopy. More than 95% of the cell preparations were viable and contained >95% macrophages.

2.4. Assessment of cell viability

Peritoneal macrophages were seeded at a concentration of 1×10^5 cells/well in 96-well tissue culture plates and treated with prodigiosin for 1 h. Cell viability was measured by quantitative colorimetric assay with MTT (3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide), showing the mitochondrial activity of living cells as previously described [29]. The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density at 550 nm using a Molecular Device microplate reader (Sunnyvale, CA).

2.5. Nitrite determination

The amount of NO_2^- accumulated in the culture supernatants was measured using the assay system reported by Ding et al. [30]. Briefly, 100 μl of the supernatant was removed from each well and placed into an empty 96-well plate. After adding 100 μl Griess reagent to each well, the absorbance was measured at 550 nm using a Molecular Device microplate reader. The NO_2^- concentration was calculated from a $NaNO_2$ standard curve. The NO_2^- levels are indicative of the amount of NO production. The Griess reagent was prepared by mixing 1 part of 0.1% naphthylethylene diamine dihydrochloride in distilled water with 1 part of 1% sulfanilamide in 5% concentrated H_3PO_4.

2.6. Reverse transcription-polymerase chain reaction (RT–PCR)

The expression of inducible nitric oxide synthase (iNOS) mRNA was determined by RT–PCR analysis. After drug treatment, the total RNA was isolated from the cultured cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The total RNA isolated was amplified by Superscript one step RT–PCR with a PLATINUM Taq kit (Invitrogen). The PCR products were run on a 1.2% agarose gel and visualized by ethidium bromide staining. GAPDH was used as the normalized control. The following primers were used to amplify iNOS and GAPDH cDNAs: iNOS-F (5′-AGA CTG GAT TTG GCT GGT CCC TCC-3′), iNOS-R (5′-AGA ACT GAG GGTACA TGC TGGAGC-3′), GAPDH-F (5′-CCATGGAGAAGGCTGGGG-3′), GAPDH-R (5′-CAAAGTTGTCATGGATGACC-3′).

2.7. Cytokine determination by ELISA

Peritoneal macrophages were pretreated with prodigiosin, followed by the addition of LPS to the cultures for indicated times. The culture supernatants were collected and the TNF-α, IL-1 and IL-6 concentration in the culture supernatants was determined using DuoSet Elisa kit (R&D System, Minneapolis, MN). Manufacturer's instructions were followed. Samples were assessed in triplicate relative to standards supplied by the manufacturer.

2.8. Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA)

Peritoneal macrophages (2×10^6 cells/ml) were suspended in RPMI 1640 medium supplemented with 10% FBS and placed in 6-well plates (3 ml/well) and incubated at 37 °C. The cells were then incubated for 1 h after first exposing them to prodigiosin before LPS stimulation and collected on ice before isolating the nuclear extracts. The cells were washed with ice-cold phosphate-buffered saline and suspended in 200 μl of a lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). The cells were then allowed to swell on ice for 15 min, after which 12.5 μl of 10% nonidet P-40 was added. The tube was mixed thoroughly for 10 sec using a Vortex mixer prior to centrifugation (10,000 ×*g*) at 4°C for 3 min. The nuclear pellets obtained were resuspended in 25 μl of an ice-cold nuclear extraction buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol), and kept on ice for 15 min with intermittent agitation. The samples were subjected to centrifugation for 5 min at 4 °C, and the supernatant was stored at −70 °C. An aliquot was taken and the protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The EMSAs were carried out using a digoxigenin (DIG) gel shift kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's protocol. Briefly, oligonucleotides 5′-AGTTGAGGGGACTTTCCCAGG-3′ and 5′-CGCTTGATGAGTCAGCCGGAA-3′ containing the NF-κB and AP-1 binding site, respectively, were DIG-labeled using a 3′-end labeling kit, and the DNA probe was incubated with 10 μg of the nuclear extract at room temperature for

10 min. Subsequently, the protein–DNA complexes were separated on a 6% polyacrylamide gel, and electrically transferred to a nylon membrane (Boehringer Mannheim Biochemica) for chemiluminescence band-detection. The specificity of the binding was examined using competition experiments, where a 100-fold excess of the unlabeled oligonucleotide with the same sequence or unrelated oligonucleotide (5′-CTAGTGAGCCTAAGGCCGGATC-3′) was added to the reaction mixture before adding the DIG-labeled oligonucleotide.

2.9. Western blot analysis

Western blot analysis was performed by a modification of a technique described elsewhere [31]. After the treatment, the cells were washed twice in PBS and suspended in a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml phenylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin). The cells were placed on ice for 30 min. The supernatant was collected after centrifugation at 15,000 g for 20 min at 40 °C. The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Lab, Hercules, CA) with BSA (Sigma) as the standard. The whole lysates (20 μg) were resolved on a 7.5% SDS–polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington heights, IL) and probed with the appropriate antibodies. The blots were then developed using an enhanced chemoluminescence (ECL) kit (Amersham). In all immunoblotting experiments, the blots were reprobed with the anti-β-actin antibody as a control for the protein loading.

2.10. Statistical analysis

Each result is reported as means±S.E.M. Two-way analysis of variance was used for analysis of differences among groups and the significant values are represented by an asterisk. ($*p<0.05$).

3. Results

3.1. Structural determination of the pigment of H. chejuensis

A maximum absorbance of the purified red pigment at 535 and 470 nm in acidic and basic conditions, respectively, suggested that the pigment is a prodigiosin-like compound. Through LC–ESI–MS/MS analysis, the fragmentation pattern of a base peak [23.73 min; m/z 324.2, $(M+H)^+$] from the red pigment was shown to be identical to that of the antibiotic prodigiosin, which was further confirmed by ^1H NMR and ^{13}C NMR analyses (Materials and methods and Fig. 1).

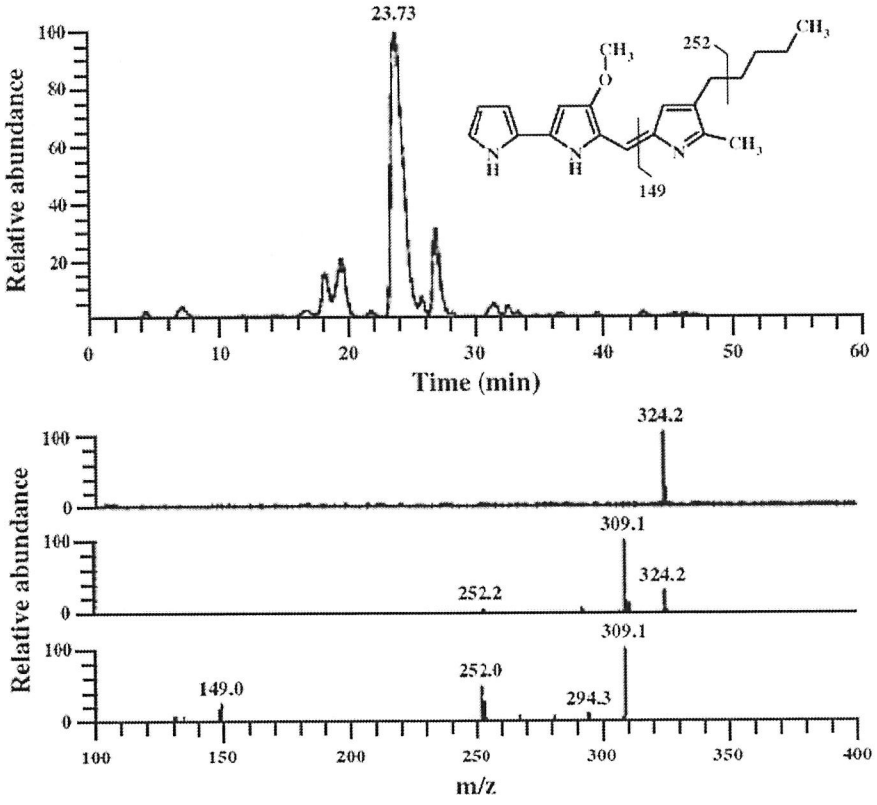

Fig. 1. Structure of the red pigment of *H. chejuensis*. Structural determination of the red pigment. Upper part, LC–ESI–MS in the positive-ion mode; lower part, MS/MS fragmentation pattern of the base peak (23.73 min).

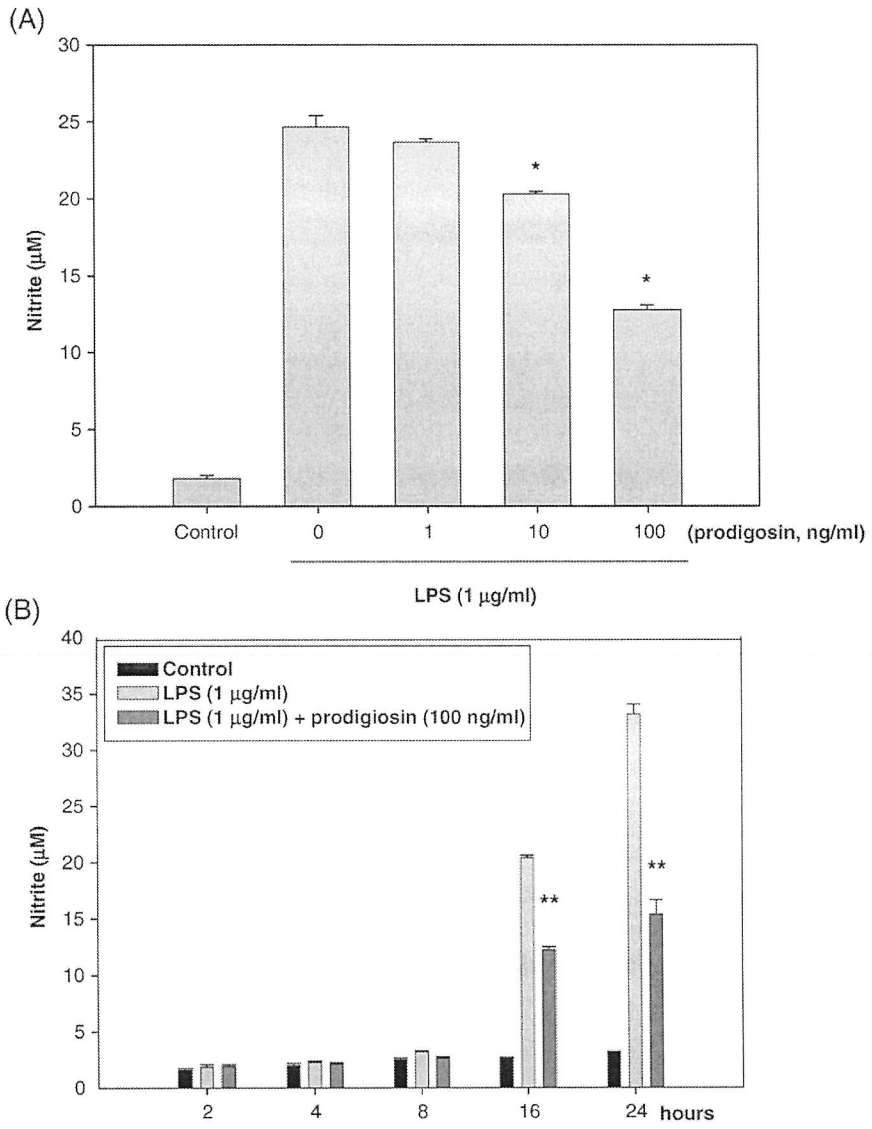

Fig. 2. Effects of prodigiosin on the LPS-induced NO production in macrophages. The cells were incubated with a medium containing various concentrations of prodigiosin for 1 h (A) before LPS treatment (1 μg/ml) and the amount of nitrite in the medium was monitored for 24 h. (B) Data are expressed as the mean ± SEM of quintuplicates from a representative experiment.* Significantly different from control (no treatment); $p<0.05$. ** Significantly different from LPS-treated; $p<0.05$.

3.2. Effect of prodigiosin on NO production and iNOS expression in LPS-stimulated macrophages

The effect of prodigiosin pretreatment on LPS-induced NO production in murine peritoneal macrophages was examined (Fig. 2A). Pretreatment of cells with prodigiosin 1 h before stimulation with LPS (1 μg/ml) resulted in reduction of NO production in a concentration-dependent manner, which reached statistical significance at 10 ng/ml. Prodigiosin did not affect the cell viability but concentrations > 100 ng/ml were found to be cytotoxic (data not shown). Macrophages were incubated with prodigiosin (100 ng/ml), followed by the addition of LPS (1 μg/ml) to the cultures for various times. Prodigiosin inhibited NO production in cells treated with LPS for 16 or more (Fig. 2B).

The iNOS mRNA and protein level were also measured to determine if the decrease in NO production by prodigiosin is due to the inhibition of iNOS formation. As shown in Fig. 3, a significant increase in iNOS expression compared with the untreated cells was observed in the LPS-treated macrophages. However, pretreatment of the cells with prodigiosin 1 h before stimulation caused a concentration-dependent reduction in iNOS mRNA expression. In addition, prodigiosin significantly decreased LPS-induced iNOS protein level. This inhibitory effect was selective, because prodigiosin did not affect the expression of GAPDH and β-actin.

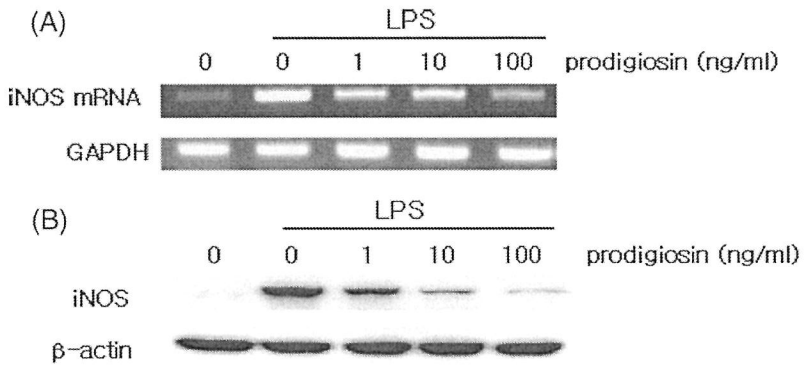

Fig. 3. Effects of prodigiosin on the expression of iNOS in LPS-stimulated macrophages. The cells were treated with prodigiosin at the concentrations indicated for 1 h followed by the stimulation with LPS at 1 μg/ml. (A) The levels of iNOS mRNA expression were determined by RT–PCR analysis. Internal control of GAPDH mRNA was also measured. (B) The iNOS protein levels were determined by Western blot assay. The β-actin protein level was considered as an internal control. The results are representative of three separate experiments.

Proinflammatory cytokines such as IL-1β, IL-6, and TNF-α is elevated in inflammatory disease and is important role in immune and inflammatory response. Therefore, the effects of prodigiosin on the levels of proinflammatory cytokines in the LPS-stimulated macrophages were examined. Prodigiosin did not affect cytokines production (IL-1β, IL-6, and TNF-α) compared with those in the LPS-treated cells (data not shown).

3.3. Effect of prodigiosin on the activation of NF-κB and AP-1 in LPS-stimulated macrophages

Because the transcription factors NF-κB and AP-1 activation are critical for the iNOS expression [32], the effect of prodigiosin on LPS-induced NF-κB and AP-1 activity was examined. When stimulated with LPS for 2 h, NF-κB activity increased by 3-fold, compared to untreated cells. Pretreatment of the cells with prodigsiosin significantly decreased the LPS-inducible increase in NF-κB activity in a dose-dependent manner(Fig. 4A). However, when cells were pretreated with prodigiosin, prodigiosin did not alter LPS-induced AP-1 transcriptional activity. These results suggest that prodigiosin inhibits the activation of NF-κB, which might be associated with the blocking of LPS-inducible iNOS expression by prodigiosin. In contrast, AP-1 may not be involved in the inhibitory actions of prodigiosin.

Since LPS-stimulated activation of NF-κB is correlated with IκBα degradation, the effect of prodigiosin on IkBα expression was examined to clarify the inhibitory action of prodigiosin (Fig. 4B). LPS stimulation caused a marked reduction in IkBα protein level within 30 min and by 120 min it reappeared, but in prodigiosin-pretreated cells LPS failed to degradation of IkBα. Taken together, these results indicate that prodigiosin inhibits both LPS-induced NF-κB activation and IkBα degradation.

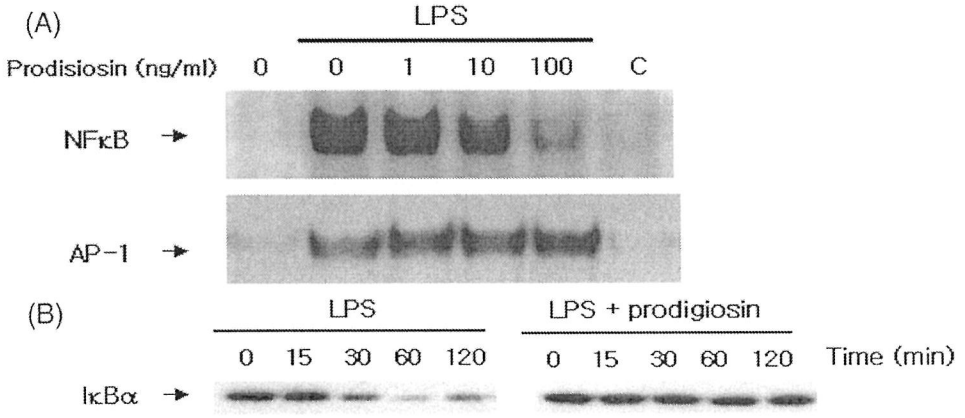

Fig. 4. Effects of prodigiosin on NF-κB and AP-1 activation and IκBα degradation in LPS-stimulated macrophages. (A) The cells were pretreated with various concentrations of prodigiosin 1 h before LPS treatment (1 μg/ml) for 2 h. Nuclear proteins (10 μg) were subjected to EMSA using the consensus binding sequence of NF-κB or AP-1. C, The binding specificity was determined using the unlabeled wild-type probe to compete with the labeled oligonucleotide. (B) The cells were pre-incubated with or without prodigosin (100 ng/ml) for 1 h, then treated with LPS (1 μg/ml) induction for the times indicated. Whole cell lysates were analyzed by Western blot with anti-IκBα antibody. The results are representative of three separate experiments.

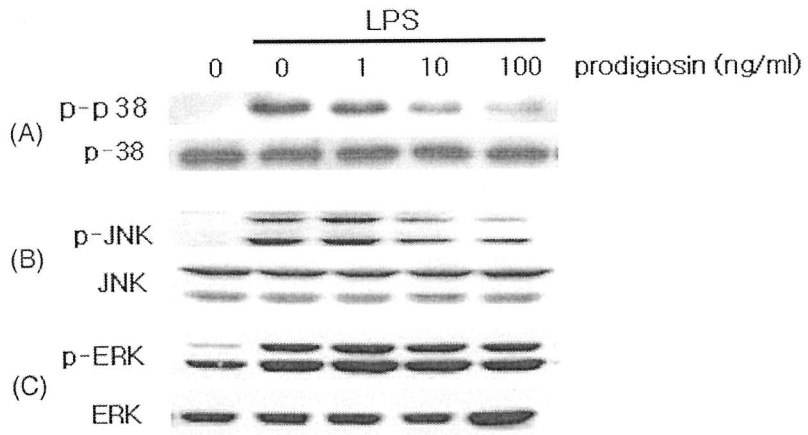

Fig. 5. Effect of prodigiosin on p-p38 (A), p-JNK (B) and p-ERK (C) activation in LPS-stimulated macropahges. Cells were pretreated with the indicated concentration of prodigiosin for 1 h and then incubated with LPS (1 μg/ml) for 20 min. The whole-cell lysates were analyzed by Western blot. The results are representative of three separate experiments.

3.4. Effect of prodigiosin on MAP kinases in LPS-stimulated macrophages

The p38 MAPK, ERK1/2 and JNK kinase pathway were examined to determine if prodigiosin interferes with the activation of macrophages by LPS (Fig. 5). This is because the MAP kinase pathways are involved in controlling the production of inflammatory mediators such as NO, and given that the prodigiosin treatment influences NO production and iNOS expression. LPS clearly stimulated an increase in the levels of activation of p38 MAPK, ERK1/2 and JNK in untreated cells. However, the induced p38 MAPK and JNK activities were inhibited by pretreatment for 1 h above 10 ng/ml concentration of prodigiosin, whereas prodigiosin did not inhibit the activation of ERK1/2. These results suggest that prodigiosin may reduce LPS-induced NO production by suppressing the phosphorylation of p38 MAPK and JNK pathway.

4. Discussion

The present study elucidate the ability and molecular mechanisms of prodigiosin in inhibition of LPS-induced NO production and iNOS gene expression in murine peritoneal macrophage. The inhibition by prodigiosin of the LPS-stimulated expressions of these molecules was not attributable to prodigiosin cytotoxicity, as assessed by MTT assay and the expression of the housekeeping genes, GAPDH and β-actin. To our knowledge, this is the first report showing the inhibitory effect of prodigiosin on macrophage function.

It was previously shown that stimulation of macrophages by LPS resulted in the expression of iNOS, which catalyzes the production of NO [33,34]. NO acts as an intracellular messenger and regulates cellular functions such as vasorelaxation and inflammation. NO has an important role in the elimination of pathogens and tumor cells. However, overproduction of NO, predominantly via upregulation of iNOS in macrophages, contributes to numerous pathological processes, including inflammation [35]. Since macrophage-associated NO production play a crucial role in the induction of tissue injury in inflammatory diseases, prevention of iNOS expression represents an important therapeutic goal. In this study, prodigiosin inhibits iNOS expression as shown by a decrease in iNOS mRNA and iNOS protein levels. These results exclude the possibility that prodigiosin affected the stability of expressed iNOS mRNA, implicating that prodigiosin acts presumably by inhibiting the transcription of iNOS gene. GAPDH mRNA levels remained unchanged, thereby excluding non-specific actions of prodigiosin on gene expression. Therefore, it seems likely that the inhibition of NO production is attributed to the suppression of iNOS mRNA transcription followed by protein expression.

It has been reported that proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α are produced by activated macrophages [12–15]. In addition, previous study showed that a decrease in nitric oxide synthesis can significantly inhibit LPS-induced IL-1, IL-6 and TNF-α expressions in macrophages [36]. However, the present study demonstrated that prodigiosin did not inhibit the IL-1, IL-6 and TNF-α levels in LPS-stimulated macrophages, suggesting that prodigiosin may have a differential effect on the production of many proinflammatory mediators by activating macrophages. It is also plausible that IL-1, IL-6 and TNF-α expressions might not be associated with the suppression of iNOS expression by prodigiosin in LPS-activated macrophages.

Protein kinases play an important role in the signal transduction pathways that regulate the response of macrophages to external stimuli including LPS [19–24]. Many independent lines of evidences suggest that macrophages may utilize different MAPK signaling pathways for LPS-inducible NO production and that the MAPK signaling pathways are differently involved in response to anti-inflammatory compounds. In the present study, our data demonstrate that prodigiosin significantly inhibited phosphorylation of p38 MAPK and JNK but not ERK1/2 phosphorylation in LPS-activated macrophages. Thus, these results suggest that prodigiosin treatment inhibits LPS-induced iNOS expression and NO production through selective inhibition of p38 MAPK and JNK activation. Moreover, the different effects of prodigiosin on NO and cytokine production can possibly be explained by the differential role of p38 MAPK and JNK in the regulation of cytokine expression.

The activation of MAPK leads to activation of transcription factors which are important molecular targets for pharmacological intervention and drug development, since they are critical in the regulation of multiple genes involved in the control of immune and inflammatory responses. Among various transcription factors, NF-κB and AP-1 regulate many important functions in macrophages and play a crucial role in expression of cytokines and NO production [37]. We found that LPS treatment increased the DNA binding activity of NF-κB and AP-1 and that prodigiosin inhibited NF-κB activation without affecting AP-1 activity.

NF-κB is present in the cytosol as a pre-formed trimeric complex. The P50/P65 protein dimer is associated with an inhibitory protein known as IκB [38]. When the cells are stimulated with extracellular stimulus, such as LPS, IκB is phosphorylated and dissociates from NF-κB, resulting in its migration to the nucleus and the activation of target gene expression. Receptor signaling leads to activation of 2 IκB kinases, IKKα and IKKβ, which phosphorylates IκB. IκBα degradation results in rapid changes in NF-κB induction, whereas IκBβ degradation is associated with prolonged NF-κB activation [39]. The present data demonstrate that LPS stimulation induced a significant decrease in IκBα, followed by gradual reappearance of IκBα, probably owing to a feedback regulation, resynthesis of IκBα. In contrast, prodigiosin inhibited LPS-induced IκBα degradation, and thereby blocked NF-κB translocation to the nucleus. Thus, this mechanism appears to be involved in the action of prodigiosin on iNOS expression in LPS-treated macrophages. Activation of MAPKs has been implicated in the activation of NF-κB through the phosphorylation of IkBα in LPS-treated cells [40]. Overall our findings indicate that prodigiosin inhibits the LPS-induced phosphorylation of p38 MAPK and JNK and the inhibition of NF-κB activation by prodigiosin may be due to inhibition of phosphorylation of p38 MAPK and JNK.

It has been known that LPS binds to the LPS binding protein (LBP) and the LBP/LPS complex subsequently binds to CD14, which triggers cell activation [18,41,42]. The interaction between the ternary membrane CD14-LPS-LBP complex and the Toll like receptor 2 (TLR2) on the cell surface initiates the intracellular signal transduction for LPS in macrophages. Therefore, these membrane proteins are expressed on the surface of the macrophages and are involved in various cell-substrate interactions. However, the exact role of these membrane proteins in the response to prodigiosin remains to be elucidated.

In conclusion, this study demonstrated that prodigiosin inhibited the production of NO induced by LPS in macrophages. This action is resulting from the suppression of p38 MAPK, JNK and NF-κB activation. In addition, as NF-κB is one of the critical transcription factors controlling the transcription of many genes associated with inflammation, the inhibition of this transcription factor by prodigiosin would be a possible therapeutic approach to the treatment of inflammatory diseases.

Acknowledgements

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