Inhibitory Effect of Erythraline on Toll-Like Receptor Signaling Pathway in RAW264.7 Cells

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Erythraline, isolated from the bark of Erythrina crista-galli which are used as Brazilian medicine plant for the treatment of inflammatory diseases, suppressed nitric oxide (NO) production and induction of inducible nitric oxide synthase (iNOS) expression in RAW264.7 cells stimulated by lipopolysaccharide (LPS). Because of Toll-like receptor (TLR) 4 and its signal transduction are indispensable to the production of NO and iNOS expression by LPS, we investigated the effects of erythraline on TLR signaling molecules. Western blot analysis revealed that the degradation of inhibitor of nuclear factor (NF)-κB (IκB) by LPS was suppressed by erythraline. Moreover, erythraline inhibited not only LPS-induced phosphorylation of IκB kinase (Ikk) but also phosphorylation of mitogen-activated protein kinases (MAPKs). However, it showed no effect on LPS-induced phosphorylation of transforming growth factor (TGF)-β-activated kinase (TAK) 1 that exists upstream of Ikk and MAPKs, and is required for the activation of these signaling molecules on TLR signaling pathway. These results suggested that erythraline might have inhibited the kinase activity of TAK1. Furthermore, these results were supported from the inhibitory pattern of erythraline on TLR signaling molecules when the cells were stimulated by TLR2 ligand, peptidoglycan which activates the same pathway as LPS on TLR signal transduction.

Key words erythraline; transforming growth factor-β activated kinase 1; anti-inflammation; toll-like receptor

The Erythrina genus (Leguminosae) includes more than 100 species and is distributed in South America and tropical and subtropical regions of South Asia. Erythrina crista-galli L. is commonly known as the cockspur coral tree. It is also called “Corticeira” in Brazil and its bark is used for the treatment of rheumatism, hepatitis, sedation, and hypnogenesis. In the course of a screening program for natural compounds showing nitric oxide (NO) inhibition activity, we found that erythraline which is Erythrinan alkaloids isolated from Erythrina crista-galli inhibited lipopolysaccharide (LPS)-induced NO production. NO is known to be an endogenous regulator of cell and tissue function and important pro-inflammatory mediator in the inflammatory process. NO is a free radical mediator in the inflammatory process. However, excessive production of NO leads to severe inflammatory responses. Therefore, understanding the mechanisms of NO production is of great importance.

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In the course of a screening program for natural compounds showing nitric oxide (NO) inhibition activity, we found that erythraline which is Erythrinan alkaloids isolated from Erythrina crista-galli inhibited lipopolysaccharide (LPS)-induced NO production. NO is known to be an endogenous regulator of cell and tissue function and important pro-inflammatory mediator in the inflammatory process. NO is a free radical mediator in the inflammatory process. However, excessive production of NO leads to severe inflammatory responses. Therefore, understanding the mechanisms of NO production is of great importance.

Toll-like receptor (TLR) 4 is indispensable for the production of NO in response to LPS. TLRs are key components of innate immunity and induce innate immune responses by recognizing invading microbial pathogens. The innate immunity is the first step in biophylaxis and other defenses against bacterial or viral infections, which occur through a multitude of inflammatory responses. However, an excessive immune response might be connected with inflammatory disorders such as sepsis and chronic inflammation. Thirteen TLRs have been discovered in mammalian cells and have been identified to have different ligands. It is described in previous studies that TLR2 recognizes zymosan and peptidoglycan, TLR4 recognizes lipopolysaccharide, TLR5 recognizes flagellin, and TLR7/8 recognizes single stranded RNA. It has been reported that TLRs relate to many intracellular signaling molecules, such as myeloid differentiation factor (MyD) 88, interleukin-1 receptor associated kinase (IRAK)-1, IRAK-4, TIR domain-containing adapter protein (TIRAP), TNF receptor associated factor (TRAF) 6, transforming growth factor (TGF)-β-activated kinase (TAK) 1, inhibitor of nuclear factor (NF)-κB (IκB), IκB kinase (Ikk), and p38, p44/42 mitogen-activated protein kinase (MAPK). The stimulation of TLRs is required for the activation of the downstream signaling pathways leading to the activation and nuclear translocation of NF-κB. The activation and translocation of NF-κB leads to the expression of its target genes, and induces the production of various inflammatory mediators such as NO, prostaglandin (PG) E2, and TNF-α.

In the present study, we investigated the effect of erythraline on LPS- and peptidoglycan (PGN)-induced TLR signal transduction. The aim of this study was to analyze the mechanism of action for the inhibitory effect of erythraline on LPS-induced NO production and to clarify the molecular target of erythraline within TLR signaling pathways.

MATERIALS AND METHODS

Plant Materials and Isolation of Erythraline Purchase and identification of the bark of Erythrina crista-galli and isolation of erythraline were described previously.

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Reagents  The polyclonal antibodies specific for IκB-α, Ikka, phospho-Ikka/β, p38 MAP kinase, phospho-p38 MAP kinase, SAPK c-Jun N-terminal kinase (JNK), phospho-SAPK/JNK, TAK1, IRAK-1 and anti-rabbit immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). The polyclonal antibodies specific for cyclooxygenase (COX)-1 and NOS2, were purchased from Santa Cruz Biotechnology (Delaware, CA, U.S.A.). Biotinylated anti-rabbit, mouse, goat IgG (H+L) and Vectastain ABC kit were purchased from Vector Laboratories (Burlington, CA, U.S.A.). The ECL Western blot detection reagent was purchased from GE Healthcare (Piscataway, NJ, U.S.A.). Lipopolysaccharides (LPS, from *Escherichia coli*, serotype 026:B6), peptidoglycans (PGN, from *Staphylococcus aureus*), and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Culture  RAW264.7 cells, a murine macrophage cell line, were obtained from Riken Cell Bank (Tsukuba, Ibaraki, U.S.A.). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), phenol red-free, supplemented with 10% fetal bovine serum (FBS; EIDIA, Tokyo, Japan), 100 U/mL penicillin (Meiji Seika, Tokyo, Japan) and 100 mg/mL streptomycin (Meiji Seika). In all experiments, the cells were grown to 80–90% confluence and subjected to no more than 15 cell passages.

Measurement of Cell Viability  Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 cells (1×10⁵ cells/well) were pre-incubated at 37°C for 3 h with or without various concentrations of erythraline (3, 10, and 30 μM). After pre-incubation, LPS (10 μg/mL) was added and cells were incubated for 20 h. After incubation MTT (5 mg/mL; Sigma) was then added, and the cells were incubated for 4 h. The culture medium was removed and the cells were dissolved in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The optical densities (OD) at 540 and 620 nm were measured using a microplate reader (Model 680, Bio-Rad Laboratories, CA, U.S.A.).

Measurement of NO  The NO concentrations in the conditioned medium were determined by using Griess reagent [1% sulfanilamide (Sigma), 0.1% N-1-naphthylenediamine dihydrochloride (Sigma), and 2.5% phosphoric acid (Kanto Chemicals, Tokyo, Japan)]. RAW264.7 cells (1×10⁵ cells/well) were pre-incubated at 37°C for 3 h with or without various concentrations of erythraline (3, 10, and 30 μM). After pre-incubation, LPS or PGN (10 μg/mL) was added and cells were incubated for 20 h. After incubation, 100 μL aliquots of medium were mixed with an equal volume of Griess reagent. The absorbance was measured at 540 nm with a microplate reader after incubation for 5 min.

Western Blot Analysis  RAW264.7 cells (2×10⁶ cells) in 6 well tissue culture dishes were plated. The cells were pre-incubated at 37°C for 3 h with or without various concentrations of erythraline and treated with LPS (iNOS, COX-2: 16h, TAK1: 5 min, IRAK-1, Ikκ-α, IκB-α, JNK and p38 MAPK: 10 min) or PGN (TAK1: 10 min, IRAK-1 and Ikκ-α: 20 min, IκB-α: 30 min). The cells were washed in cold phosphate-buffered saline (PBS), and 50 μL of lysis buffer (20 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES) pH 7.3, 1% Triton-X, 10% glycerol, 1 mM sodium fluoride, 1 mM Na₃VO₄, and 10% protease inhibitor cocktail) was added to each well. After 15 min incubation on ice, the cells were collected into sample tubes by silicon policeman and incubated for 10 min on ice. The cells were then centrifuged at 15490×g for 20 min at 4°C. The supernatant fractions were boiled for 5 min in sample buffer [50 mM Tris, pH 7.4, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 4% 2-mercaptoethanol, and 0.05 mg/mL bromophenol blue], and stored at −80°C. Preparation of nuclear extracts was performed, as follows. RAW264.7 cells (2×10⁶ cells/well) were plated in 10 cm tissue culture dishes. The cells were pre-incubated at 37°C for 3 h with or without various concentrations of erythraline and treated with LPS for 15 min. At the end of incubation, the cells were harvested by scraping, followed by centrifugation at 1500 rpm and 4°C for 5 min. After centrifugation, the cell pellets were resuspended in 800 μL of Tris-buffered KCl solution (20 mM Tris–HCl, pH 7.8, 50 mM KCl) supplemented with 1 mM dithiothreitol, 50 mM sodium fluoride, 1 mM Na₃VO₄ and protease inhibitor cocktail. Nonident P-40 substitute (Wako) was added at 0.6% final concentration, and the cell suspension was vortexed for 10 s and centrifuged at 15490×g for 1 min at 4°C to obtain the nuclei pellet. The nuclei were resuspended in 30 μL ice-cold Tris-buffered high-KCl solution (20 mM Tris–HCl, pH 7.8, 500 mM KCl) supplemented with 20% glycerol, 1 mM dithiothreitol, 50 mM sodium fluoride, 1 mM Na₃VO₄ and protease inhibitor cocktail. After 1 h incubation on ice, the nuclear lysate was centrifuged at 15490×g for 20 min at 4°C, and the supernatant (nuclear extract) was mixed with 3× sample buffer (50 mM Tris, pH 7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol and 0.05 mg/mL bromophenol blue) at 2:1 ratio (v/v), boiled for 5 min, and stored at −80°C. The proteins were separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer (20 mM Tris, pH 8.3, 150 mM glycine, and 20% MeOH). The PVDF membrane was then blocked by incubation in 20% Block Ace (DS Pharma Biomedical, Osaka, Japan). Subsequently, the membrane was incubated with the antibody for overnight at 4°C. After incubation, the membrane was washed and incubated for 4 h at 4°C with a secondary antibody. Then, the membrane was incubated for 30 min at room temperature with ABC reagent. The protein bands were visualized using an enhanced chemiluminescence system.

Statistical Analysis  The data were expressed as the mean±S.E.M. of the results obtained from a number of experiments. The statistical significance of the results was analyzed by Student’s t-test.

RESULTS

Effects of Erythraline on Cell Viability  To confirm that the inhibitory effect of erythraline on NO production was not because of cytotoxicity, we examined its effects on cell viability. RAW264.7 cells (1×10⁶ cells/well) were pre-incubated at 37°C for 3 h with or without various concentrations of erythraline (3, 10, and 30 μM) and LPS (10 μg/mL). In these conditions, the cytotoxicity of erythraline was evaluated with MTT assay, but cell viabilities were not affected (Fig. 1B).

Effects of Erythraline on LPS-Induced NO Production and iNOS Protein Induction  When RAW264.7 cells were incubated at 37°C for 20 h in medium containing LPS (10 μg/
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mL), NO production was strongly increased (Fig. 2B). In the presence of erythraline (3 h pre-treatment), NO production stimulated by LPS was inhibited at 3–30 µm in a concentration-dependent manner (Fig. 2B). Western blot analysis revealed that iNOS levels at 16 h were increased by LPS. Under the condition, erythraline inhibited LPS-induced iNOS protein levels at 3–30 µm in a concentration-dependent manner (Fig. 2A). In contrast, COX-1 protein levels which constitutively expressed in RAW264.7 cells did not change following treatment with LPS when determined at 16 h (Fig. 2A). Treatment with 3–30 µm of erythraline in the presence of LPS also did not affect the protein levels of COX-1 at 16 h (Fig. 2A).

Effects of Erythraline on LPS-Induced NF-κB Nuclear Translocation Activation of NF-κB is known to be required for iNOS expression. Therefore, we investigated the translocation of p65, a subunit of NF-κB, from the cytosol to the nucleus by western blot analysis. RAW264.7 cells were pretreated for 3 h with 30 µm of erythraline before LPS was added. Incubation of RAW264.7 cells at 37°C in medium containing LPS for 15 min increased p65 protein levels in nuclear extracts, indicating that NF-κB is translocated into nucleus on treatment with LPS (Fig. 3). In the presence of erythraline, the extent of translocation of NF-κB into nucleus on treatment with LPS was suppressed. On the other hand, erythraline did not affect the protein levels of Oct-1, for maintaining normal physiological functions in nucleus (Fig. 3).

Effects of Erythraline on LPS-Induced TLR Signaling Transduction Factors To determine whether erythraline inhibited TLR signal transduction, cell lysates were prepared.

Fig. 1. The Chemical Structure of Erythraline (A) and Effects of Erythraline on Cell Viability in RAW264.7 Cells (B)

RAW264.7 cells (1×10⁵ cells) were pre-treated at 37°C for 3 h with the indicated concentrations of erythraline before LPS (10 µg/mL) was added, and the cells were incubated for 20 h. Cell viability was measured by MTT assay. LPS-treated control group is set to 100%. The results shown are representatives of three independent experiments.

Fig. 2. Effects of Various Concentrations of Erythraline on NO Production and iNOS Expression in LPS-Stimulated RAW264.7 Cells

RAW264.7 cells (2×10⁶ cells) were pre-treated at 37°C for 3 h with the indicated concentrations of erythraline (3–30 µm) before LPS (10 µg/mL) was added, and the cells were incubated for 16 h. The protein levels of iNOS and COX-1 were determined by Western blot analysis (A). The relative density ratios of iNOS protein to COX-1 protein are shown as histograms. The density ratio of LPS control is set to 100. Results shown are representatives of three independent experiments. Statistical significance; **p<0.01 vs. corresponding LPS control.

Fig. 3. Effects of Erythraline on LPS-Induced Nuclear Translocation of NF-κB (p65) in RAW264.7 Cells

RAW264.7 cells (2×10⁶ cells) were pre-treated at 37°C for 3 h with 30 µm of erythraline before LPS (10 µg/mL) was added, and cells were incubated for 15 min (LPS). After incubation, protein levels of NF-κB (p65) and Oct-1 in the nuclear extract samples were determined by Western blot analysis. The relative density ratios of NF-κB protein to Oct-1 protein are shown as histograms. The density ratio of LPS control is set to 100. Results shown are representatives of three independent experiments.
RAW264.7 cells were pre-treated with erythraline (30 μM) for 3 h and then incubated in the presence or absence of LPS (10 μg/mL) for the different times (Fig. 4). When RAW264.7 cells were incubated at 37°C for each time in a medium containing LPS, TLR signal transduction factors, such as TAK1, Ikk-α, IκB-α, JNK, and p38 MAPK. After incubation, each of the signal proteins and their phosphorylation or degradation forms were determined by Western blot analysis. The density of IκB-α and IκB-α protein and the relative density ratio of phosphorylated each signal proteins (TAK1, Ikk-α, JNK, and P38MAPK) to total each signal proteins are shown as histograms. The density ratio of LPS control is set to 100. Results shown are representatives of three independent experiments.

**DISCUSSION**

During our recent screening for an inhibitor of LPS-induced NO production in RAW264.7 cells, we isolated erythraline from *Erythrina crista-galli*. In the present study, we pharmacologically analyzed its mechanism of action in TLR signaling pathways. Firstly, we should mention that erythraline at 30 μM inhibited PGN-induced IκB-α degradation and Ikk-α/β phosphorylation and did not affect PGN-activated TAK-1 phosphorylation and IκB-α degradation (Fig. 5A). Under these conditions, erythraline at 30 μM inhibited PGN-induced IκB-α degradation and Ikk-α/β phosphorylation and did not affect PGN-activated TAK-1 phosphorylation and IκB-α degradation (Fig. 5A).
We found that erythraline did, however, inhibit not only LPS-induced NO production but also iNOS expression in RAW264.7 cells at 3, 10, and 30 µM in a concentration-dependent manner (Fig. 2). It has been reported that the induction of NO production stimulated by LPS operates via TLR4 signaling pathway in inflammatory cells. As a result, we proposed that anti-inflammatory effect of erythraline may operate in TLR-dependent manner and that the molecular target of erythraline is within the TLR signaling pathway. Therefore, we designed an experiment to investigate the effect of erythraline on TLR signaling.

Next, we investigated the effect of erythraline on nuclear translocation of NF-κB. NF-κB is transcription factor that activates the expression of many genes involved in the inflammatory response, including TNF-α, iNOS, and IL-1. Our result showed that erythraline inhibited the nuclear translocation of NF-κB (Fig. 3), which is likely the mechanism for the effect of erythraline on expression of iNOS. NF-κB is normally present in the cytosol in an inactive state complexed with IκB. Its activation occurs via the phosphorylation of IκB-α followed by its proteasomal degradation, which results in the nuclear translocation of NF-κB. Our results also showed that erythraline inhibited the degradation of IκB-α (Fig. 4).

The phosphorylation of Iκk is required for the degradation of IκB-α by TLR ligands. In this study, erythraline inhibited the phosphorylation of Iκk (Fig. 4).
the target molecule of erythraline was IkB or its upstream signaling components. The component upstream of IkB in the TLR signaling pathway is TAK1. Therefore, we examined the effect of erythraline on the phosphorylation of TAK1. In this study, the phosphorylation of TAK1 was evaluated by mobility shift.20) Our results showed that erythraline did not inhibit the phosphorylation of TAK1 (Fig. 4). Signal components, such as TRAF6 and IRAKs, exist upstream of TAK1 in the TLR signaling pathway. IRAKs that have been identified, such as IRAK-1,21) IRAK-2,22) IRAK-4,23) and IRAK-M,24) are important mediators in MyD88-dependent pathway of TLR signaling, in particular IRAK-4 and IRAK-1, since they possess intrinsic serine/threonine kinase activity. In addition, it has been reported that IRAK-1 is autophosphorylated and degraded after phosphorylation by IRAK-4.25) Our study showed that erythraline did not inhibit LPS-induced IRAK-1 degradation (Fig. 4). This result suggests that erythraline inhibited the signal downstream from TAK1 in the TLR signaling pathway.

Our results suggested that erythraline inhibited the phosphorylation of IkB or TAK1 kinase activity. In order to clarify the molecular target of erythraline, we looked at the effect of erythraline on MAPKs. It has been reported that MAPKs, such as JNK and p38MAPK, are phosphorylated by activated TAK1 in the TLR signaling pathway.20) Our study showed that erythraline inhibited LPS-induced phosphorylation of JNK and p38MAPK (Fig. 4). These results suggested that erythraline is able to show its anti-inflammatory effect through the suppression of TAK1 kinase activity. In addition, we examined the effect of erythraline on PGN, a ligand of TLR2-induced NO production and TLR signaling. Our results showed that erythraline inhibited NO production, degradation of IkB-α and phosphorylation of IkB (Fig. 5). Moreover, the phosphorylation of TAK1 and the degradation of IRAK-1 were not inhibited (Fig. 5). The inhibitory pattern of erythraline on TLR signaling molecules stimulated by PGN was similar to that during stimulated by LPS.

These findings suggest that TAK1 is one of the target molecules of erythraline likely involved in its anti-inflammatory effect. TAK1 is a mitogen-activated protein kinase kinase kinase that can be activated not only by TLR ligands but also by IL-1.27) It is possible that TAK1 has a role in various inflammatory reactions. Therefore, erythraline contained in Erythrina crista-galli may show the effect on inflammatory diseases, such as rheumatism and hepatitis, through inhibition of TAK1.

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