Inhibition of lipopolysaccharide and interferon-gamma-induced expression of inducible nitric oxide synthase and tumor necrosis factor-alpha by Lithospermi radix in mouse peritoneal macrophages

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Abstract

Lithospermi radix (LR, root of Lithospermum erythrorhizon Siebold et Zuccarinii) has been used to treat various conditions, such as septic shock, eczema and burns. In this study, the effect of LR on lipopolysaccharide (LPS) and recombinant interferon-gamma (rIFN-γ)-induced production of nitric oxide (NO) and tumor necrosis factor (TNF)-alpha were examined using mouse peritoneal macrophages. At 0.01–1 mg/ml, LR inhibited the LPS/rIFN-γ-induced expression of inducible nitric oxide synthase (iNOS) and TNF-alpha release. To clarify the mechanism involved, the effect of LR on the activation of nuclear factor (NF)-kappaB was examined. The LPS/rIFN-γ-induced activation of NF-kappaB was almost completely blocked by LR at 1 mg/ml without cytotoxicity. These findings demonstrate that the inhibition of the LPS/rIFN-γ-induced production of NO and TNF-alpha by LR involves the inhibition of NF-kappaB activation.

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1. Introduction

Lithospermi radix (LR, Borraginaceae, root of Lithospermum erythrorhizon Siebold et Zuccarinii) is a herbal medicine which has been used in traditional oriental medicine to treat septic shock, smallpox, eczema and other maladies. LR was reported to restore immunosuppression induced by the anti-tumor agent, cyclophosphamide (Jin et al., 1994). The extract also significantly inhibited mutagenic effects of the carcinogen N-butyl-N-butanolnitrosoamine (BBN) by suppression of chemotactic activity and production of IL-1 and tumor necrosis factor (TNF)-alpha by macrophages (Jin and Kurashige, 1996).

Nitric oxide (NO) is a highly reactive molecule produced from a guanidino nitrogen of arginine by NO synthase (NOS) enzymes (Nathan, 1992). Over the past decade, NO has received increasing attention as a potent macrophage-derived effector molecule against a variety of bacteria, parasites and tumors (Gantt et al., 2001). However, inappropriate activation of macrophages leads to excessive NO secretion, which has been implicated in the pathogenesis of various inflammatory diseases, such as rheumatoid arthritis and septic shock. Therefore, proper control of macrophage activity is
an important strategy in the treatment of chronic inflammatory diseases. TNF-α is one of the most important proinflammatory cytokines and is produced mainly by activated monocytes and macrophages (Tracey and Cerami, 1994). It induces various biological responses, including tissue injury, shock and apoptosis (Tracey et al., 1987; Baker and Reddy, 1998). TNF-α also induces the secretion of cytokines (such as interleukin (IL)-1, IL-6 and IL-10), as well as the activation of T cells and other inflammatory cells (Vilcek and Lee, 1991). Therefore, suppression of NO and TNF-α production by activated macrophages should be useful in the treatment of inflammatory diseases. Macrophages are major sources of cytokines, such as TNF-α; the induction of cytokine gene expression by lipopolysaccharide (LPS) occurs primarily at the transcriptional level. This involves the action of several transcription factors, including members of the nuclear factor-κB (NF-κB) family (Tracey, 1997; Baker and Reddy, 1998). NF-κB is also a key player in the regulation of TNF-α production. We obtained LR (Lactobacillus plantarum) from the Korean Food Research Institute and isolated it as described previously (Lin and Lin, 1997). Using 8 ml of DMEM, peritoneal lavage was performed. Cells were then distributed in DMEM supplemented with 10% heat-inactivated FBS in 24-well tissue culture plates (2.5 × 10⁵ cells/well), incubated for 3 h at 37 °C in an atmosphere of 5% CO₂, washed three times with DMEM to remove non-adherent cells and equilibrated with DMEM that contained 10% FBS before treatment.

2.3. Preparation of LR

We obtained the spray-dried LR extract from Sunten Pharmaceutical Co. (Taipei, Taiwan, lot number: 302208). LR was deposited in the Department of Physiology, College of Oriental Medicine, Kyung Hee University. One gram of LR powder contained 0.69 g of LR extract and 0.31 g of starch. We dissolved 1 g of LR powder in 0.69 ml of distilled water, centrifuged at 15,000 rpm for 10 min, transferred the supernatant to another tube and filtered through a 0.2 μm syringe filter. To authenticate LR, we performed HPLC and electron impact MS analyses. The 1 g of LR powder contained 13.28 ± 2.04 mg of shikonin (M.W.: 288) and 59.57 ± 6.73 mg of acetylshikonin (M.W.: 330.34) (data not shown).

2.4. MTS assay

Cell growth was measured by MTS assay using the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA). Briefly, 100 μl of the supernatant was added to each 96-well plate. Twenty microtiter of the MTS solution was added to each of the 96-well plates and incubated at 37 °C in a humidified 5% CO₂ atmosphere for 1 h. The absorbance was read at 490 nm using a microplate reader (Molecular Device, Sunnyvale, CA, USA). This was repeated for each sample.

2.5. Measurement of nitrite concentration

Peritoneal macrophages (2.5 × 10⁶ cells/well) were treated with various concentrations of LR. The cells were then cultured in DMEM supplemented with 10% heat-inactivated FBS in 24-well tissue culture plates (2.5 × 10³ cells/well) and incubated for 48 h. NO synthesis in cell cultures was measured by a microplate assay method, which was previously described (Xie et al., 1992). To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(l-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 540 nm was determined in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). NO₃⁻ was determined using sodium nitrite as a standard. The cell-free medium itself contained 5-8 μM of NO₃⁻; this value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.
2.6. Assay of TNF-α release

Peritoneal macrophages (2.5 × 10⁵ cells/well) were treated with various concentrations of LR. The cells were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml) and incubated for 24 h. TNF-α release was detected by OptEIA calibrated according to the manufacturer’s instructions.

2.7. Preparation of nuclear extracts

Nuclear extracts were prepared essentially according to Schreiber et al. (Schreiber et al., 1990). Briefly, dishes were washed with ice-cold PBS. The dishes were then scraped and transferred to microtubes. Cells were allowed to swell by adding 100 μl lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride). The tubes were vortexed to disrupt cell membranes. The samples were incubated for 10 min on ice and centrifuged for 5 min at 4 °C. Pellets containing crude nuclei were resuspended in 50 μl extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and incubated for 30 min on ice. The samples were centrifuged at 15,800 × g for 10 min to obtain the supernatant containing nuclear extracts. Extracts were stored at −70 °C until use.

2.8. Western blot analysis

LR-pretreated peritoneal macrophages (5 × 10⁶ cells/well) were incubated for 6 h with rIFN-γ (20 U/ml). Cells were then stimulated with LPS (10 μg/ml) for 12 h. Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature, then incubated with anti-iNOS or anti-p65 antibodies. After washing in PBS-Tween-20 three times, the blot was incubated with secondary antibody for 30 min; the antibody-specific proteins were then visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp., Newark, NJ).

2.9. Statistical analysis

Results were expressed as the mean ± S.E.M. of independent experiments. One-way analysis of variance (ANOVA) and Tukey’s test were used for the comparison between groups; the differences were considered to be significant at p < 0.05.

3. Results

3.1. Effects of LR on NO inhibition in activated peritoneal macrophages

To investigate the effects of LR, cell viability was monitored by using the MTS assay. The highest concentration of LR which did not affect mouse peritoneal macrophage viability was 1 mg/ml (Fig. 1). The levels of NO were then examined by co-culturing various concentrations of LR with LPS/rIFN-γ-stimulated peritoneal macrophages. As shown in Fig. 2, production of NO increased with LPS treatment (10 μg/ml) and rIFN-γ (20 U/ml), while LR strongly inhibited the LPS/rIFN-γ-induced production of NO.

3.2. Effects of LR on LPS/rIFN-γ-activated iNOS expression

The LPS/rIFN-γ-induced iNOS expression was suppressed by LR in a concentration-dependent manner (Fig. 3). These findings indicate that LR inhibits the LPS/rIFN-γ-induced expression of NO through inhibition of iNOS expression.

3.3. Effects of LR on LPS/rIFN-γ-induced TNF-α production

To further investigate the effects of LR on the other immunological functions of macrophages, we measured the cytokine productions from activated macrophages in the
Fig. 4. Effect of LR on the production of TNF-α by LPS/rIFN-γ-activated peritoneal macrophages. Peritoneal macrophages (2.5 × 10^5 cells) treated with various concentrations of LR were primed for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 24 h. The amount of TNF-α secreted by peritoneal macrophages was measured by ELISA. Values are the mean ± S.E.M. of over three independent experiments performed in duplicate in each run; *p < 0.005, **p < 0.005 compared to LPS/rIFN-γ.

Fig. 5. Effect of LR on the expression of p65 by LPS/rIFN-γ-activated peritoneal macrophages. Peritoneal macrophages (8 × 10^6 cells) treated with 1 mg/ml of LR were primed for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 1 h. Nuclear extracts were prepared and analyzed for p65 expression by Western blotting as described in Section 2.1, control; 2, rIFN-γ + LPS; 3, rIFN-γ + LPS + LR (1 mg/ml).

These findings indicate that the inhibition of the LPS/rIFN-γ-induced production of NO and TNF-α by LR is induced through the suppression of the LPS/rIFN-γ-induced activation of NF-κB.

4. Discussion

We demonstrated that LR reduces the production of NO and the expression of iNOS protein in LPS/rIFN-γ-treated mouse peritoneal macrophages with dose dependence. In addition, LR inhibited TNF-α secretion in a dose-dependent manner in LPS/rIFN-γ-treated mouse peritoneal macrophages. To clarify the mechanism of action of LR in the inhibition of the LPS/rIFN-γ-induced production of NO and TNF-α, the effect of LR on the activation of NF-κB, an essential transcription factor for the
expression of iNOS (Lowenstein et al., 1993) and TNF-α (Yao et al., 1997), was examined. Our findings suggest that LR blocked the LPS/rIFN-γ-induced translocation of NF-κB by preventing the degradation of IκB-α, an inhibitor of NF-κB activation (Ghosh and Baltimore, 1990), thus, inhibiting the LPS/rIFN-γ-induced production of NO and TNF-α.

Our results suggest that the effect of LR was associated not only with iNOS modulation, but also with the cytokine production in activated macrophages. In recent years, overproduction of NO has been correlated to oxidative stress (Sies and Meldrum, 1986; Ji et al., 1999) and the pathophysiology of various diseases, including arthritis, septic shock, autoimmune diseases and chronic inflammation (Moncada et al., 1991; Arteel et al., 1999). Inhibition of NO synthesis is, therefore, a potential therapeutic approach for the treatment of these inflammatory diseases. In addition to many synthetic inhibitors of iNOS, natural products which inhibit NO production have been investigated (Chiou et al., 1997; Kobuchi et al., 1997; Ryu et al., 1998; Raso et al., 2001). On the other hand, an excessive amount of TNF-α has also been implicated in the pathogenesis of many chronic inflammatory diseases (Firestein et al., 1994). Because of its pivotal role in pathogenesis, a significant effort has been focused on developing drugs that interfere with TNF-α production or action (Moreira et al., 1993; Cohun et al., 1996).

In terms of a possible inhibitory mechanism, cell viability was not altered by LR, indicating that inhibition of NO and cytokine production is not due to a cytotoxic effect. In macrophages, NF-κB (in cooperation with other transcription factors) coordinates the expression of genes encoding iNOS (Spink et al., 1995; Pan et al., 2000; Mandrika et al., 2001) and plays a critical role in the activation of immune cells by up-regulating the expression of many cytokines, including TNF-α (Kopp and Ghosh, 1995). Previous studies had shown that several natural products potently suppress NO and TNF-α production from macrophages by inhibiting NF-κB activation induced by LPS signal (Baeuerle and Henkel, 1994; Chen et al., 2000). Shikonin, the naphthoquinone pigment, is a major constituent of LR. Shikonin had been reported as having radical-scavenging and anti-inflammatory effects (Kourounakis et al., 2002; Stanforth et al., 2004). Therefore, we assumed that the effects of LR in this study would be related with shikonin or its analogues.

In conclusion, this study showed that LR suppressed the LPS/rIFN-γ-induced production of NO and TNF-α in peritoneal macrophages through inhibition of NF-κB activation, although the precise mechanism of LR to inhibit NO and TNF-α production remains to be further elucidated. Since NF-κB is a critical transcription factor which regulates the production of various proinflammatory proteins and cytokines in activated macrophages during the process of inflammation, the inhibition of this transcription factor might serve as an effective therapeutic approach for inflammatory diseases, such as rheumatoid arthritis.

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References


