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Suppressive effects of *Lithospermum erythrorhizon* extracts on lipopolysaccharide-induced activation of AP-1 and NF-κB via mitogen-activated protein kinase pathways in mouse macrophage cells

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A variety of anti-inflammatory agents have been shown to exert chemopreventive activity via targeting of transcription factors such as NF-κB and AP-1. *Lithospermum erythrorhizon* (LE) has long been used in traditional oriental medicine. In this study, we demonstrated the inhibitory effects of LE extracts on lipopolysaccharide (LPS)-stimulated production of inflammatory cytokines. As an underlying mechanism of inhibition, LE extracts reduced LPS-induced transactivation of AP-1 as well as NF-κB in mouse macrophage cells. Electrophoretic mobility shift assays indicated that LE extracts inhibited the DNA binding activities of AP-1 and NF-κB. In addition, phosphorylation of IκB-α protein was suppressed by LE extracts. Moreover, LE extracts inhibited c-Jun N-terminal kinase and extracellular signal-regulated signaling pathways. Our results suggest that the anti-inflammatory activity of LE extracts may be mediated by the inhibition of signal transduction pathways that normally lead to the activation of AP-1and NF-κB. These inhibitory effects may be useful for chemoprevention of cancer or other chronic inflammatory diseases. [BMB reports 2008; 41(4): 328-333]

INTRODUCTION

Inflammation and carcinogenesis are known to be related phenomena, and many studies have attempted to link these two processes based on their mechanisms (1). It has long been suggested that chronic inflammation is involved in a variety of cancers such as lung and stomach cancer (2, 3). The inflammatory response is accompanied by an upregulation of cytokines, growth factors, inducible NO synthase (iNOS) and many other enzymes and factors (4-6) that may contribute to the initiation and promotion of tumor formation. Pro-inflammatory genes, such as TNF-α and iNOS are induced by a wide range of signals, including lipopolysaccharides (LPS), phorbol esters and growth factors, through the activation of AP-1 and NF-κB (7, 8).

AP-1 and NF-κB are transcription factors associated with carcinogenesis as well as inflammation (9-11). Activation of AP-1 is associated with neoplastic transformation, and expression of mutant c-Jun or blocking 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced AP-1 activation has been shown to inhibit malignant transformation (12, 13). NF-κB also plays an important role in cell proliferation, apoptosis, and tumorigenesis. Studies *in vivo* and *in vitro* indicate that control of apoptosis by NF-κB is critical to its promotion of oncogenesis (9, 10). Activation of mitogen-activated protein kinase (MAPK) signaling pathways, such as the extracellular signal-regulated kinases (ERK) 1/2, c-Jun amino-terminal kinases (JNKs), and the p38 MAP kinases, converge on stimulation of AP-1 and NF-κB (14, 15).

Lithospermum erythrorhizon (LE) has long been used in traditional Asian medicine for the treatment of skin measles, chicken pox, hepatitis and skin cancer. It has been reported that extracts from the roots of LE restored immunosuppression induced by cyclophosphamide, an anti-tumor agent (16). Additionally, LE extracts have been found to suppress LPS- and IFN-γ-induced production of inducible NO synthase (iNOS) and TNF-α by macrophages (17) and to inhibit the mutagenic effects of the carcinogen, N-butyl-N-butanolnitrosamine (18). It has also been reported that LE extracts exhibited inhibitory effects on *Trypanosoma cruzi* and HIV (19, 20).

Little information regarding the underlying mechanisms of LE extracts at the cellular and molecular levels is available. In the present study, we investigated the inhibitory effect of LE extracts on cytokine expression in LPS-stimulated macrophage cells and the underlying mechanism of this inhibition. We demonstrated that LE extracts inhibited the activities of AP-1

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and NF-κB. In addition, we showed that inhibition of LPS-induced activation of JNK and p38 MAPK signaling pathways by LE extracts may be the underlying cause of the reduced transactivation and DNA binding activities of AP-1 and NF-κB.

RESULTS

Inhibitory effect of LE extracts on LPS-induced expression of inflammatory cytokines

In order to gain insight regarding the mechanism(s) by which LE extracts affect the production of inflammatory cytokines in RAW264.7 macrophage cells, we investigated the effects of LE extracts on the LPS-induced increase in mRNA levels of various cytokines. The results of our RT-PCR analyses indicated that the LE extracts effected a concentration-dependent reduction in IFN-γ, TNF-α, IL-6, iNOS, and IL-1β mRNA levels in LPS-stimulated cells, without affecting the mRNA expression of GAPDH, a housekeeping gene (Fig. 1A). Quantitative real-time PCR analysis confirmed that LE extracts led to a dose-dependent decrease in mRNA levels of the anti-inflammatory cytokines in the LPS-stimulated macrophage cells (Fig. 1B). Production of the cytokines was increased in LPS-stimulated cells as detected by specific ELISA. The protein levels of IFN-γ, TNF-α, IL-6, and IL-1β in the supernatants of the stimulated cells were reduced by LE extracts in a dose-dependent manner with IC_{50} values of about 45 μg/ml, 32 μg/ml, 42 μg/ml, and 23 μg/ml, respectively (Fig. 1C). Expression of iNOS mRNA was significantly reduced by LE extracts (Fig. 1A), which is consistent with previously reported results (17). The inhibitory effects of methanol extracts of LE were more potent than those of water extracts (Data not shown). LE extracts were not toxic to the RAW264.7 cells at the concentrations used in this study as previously reported (17).

Suppression of LPS-induced activation of AP-1 and NF-κB by LE extracts

In order to elucidate the mechanisms of the inhibition of cytokine gene induction by LE extracts, we examined the effects of LE extracts on the activation of a transcription factor, AP-1, which is known to be involved in the regulation of a variety of cytokine genes. To investigate the effects of LE extracts on the LPS-induced activation of AP-1, we performed AP-1 reporter assays (Fig. 2A). Our results showed that LE extracts inhibited the AP-1-dependent activation of the reporter genes with an IC_{50} value of 69 μg/ml (Fig. 2A). It has been previously reported that LE extracts inhibited NF-κB activation (17), which was confirmed using a luciferase reporter assay (Fig. 2B). The IC_{50} value for NF-κB inhibition by LE extracts was determined to be 49 μg/ml (Fig. 2B). Our results suggest that the inhibition of cytokine gene induction by LE extracts may be through inhibitory effects of the extracts on AP-1 and NF-κB functions.

Effect of LE extracts on LPS-induced DNA binding activities of AP-1 and NF-κB

To investigate whether LE extracts inhibit the DNA binding activities of AP-1 and NF-κB, nuclear extracts from LPS-treated macrophage cells with or without LE extract pretreatment were analyzed using electrophoretic mobility shift assays (EMSA). RAW264.7 cells stimulated with LPS strongly induced the DNA binding activities of AP-1 and NF-κB (Fig. 3A and 3B). The specificity of binding was examined based on competition with unlabeled specific and mutant oligonucleotides (Fig. 3A and 3B). LE extracts attenuated the LPS-stimulated DNA binding of AP-1 and NF-κB.

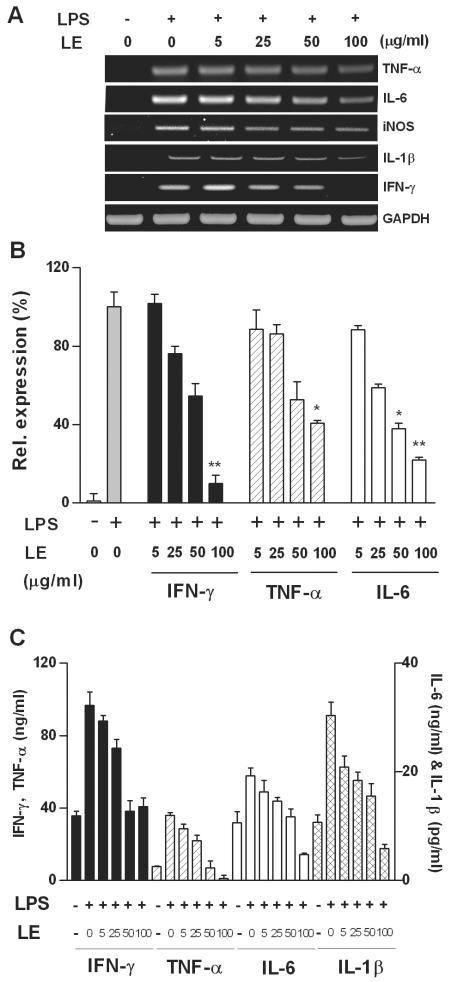

Fig. 1. Effect of LE extracts on expression of cytokines in LPS-stimulated cells. RAW264.7 cells were treated with LPS (1 μg/ml) in the presence of various concentrations of *Lithospermum erythrorhizon* extracts for 24 hrs. (A) Levels of IFN-γ, TNF-α, IL-6, iNOS, and L-1β mRNAs were determined by RT-PCR analysis. GAPDH mRNA was used as an internal control. (B) Levels of IFN-γ, TNF-α, and IL-6 mRNA were measured by real-time PCR. LPS-induced mRNA levels were inhibited by LE extracts in a dose-dependent manner. GAPDH mRNA was used as an internal control. (C) The protein levels of IFN-γ, TNF-α, IL-6 and L-1β in the supernatants were determined by ELISA analysis. Data shown are the mean values ± SD (n = 3). *P < 0.05 or **P < 0.01 compared to treatment with LPS alone.

Effect of LE extracts on LPS-induced nuclear translocation of NF-κB and AP-1

Translocation of transcription factors into the nucleus is believed to be an essential event in the activation of gene-specific binding sites. The nuclear levels of the NF-κB subunit, p65, and the AP-1 component, c-Jun, increased markedly within one hour of LPS stimulation. LE extracts resulted in a concentration-dependent decrease in the nuclear translocation of p65 and c-Jun (Fig. 4A). Activation of the NF-κB pathway involves the degradation of NF-κB inhibitors (IκBs) by the 26S proteasome (21), thereby allowing NF-κB transcription factors to translocate to the nucleus. Upon LPS activation, IκB, an inhibitor of NF-κB in the cytoplasm, is phosphorylated and subsequently degraded by the 26S proteasome, leading to the nuclear translocation of NF-κB transcription factors. LPS-stimulated phosphorylation and degradation of IκBα was inhibited in a concentration-dependent manner by LE extracts. Our results indicated that LE extracts inhibited the translocation of NF-κB into the nucleus via suppression of IκBα degradation (Fig. 4B).

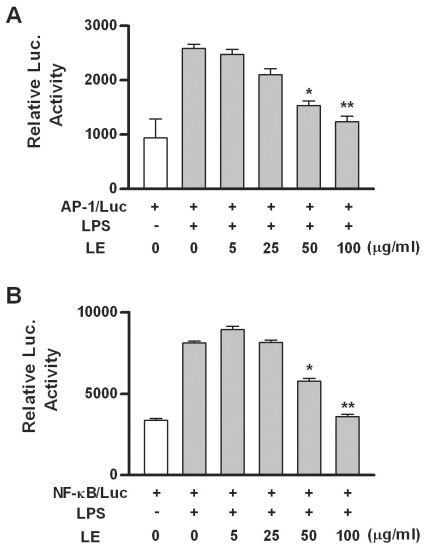

Fig. 2. The effect of LE extracts on LPS-induced expression of luciferase reporter genes. RAW264.7 cells were transiently transfected with an AP-1-dependent (A) or NF-κB-dependent reporter gene (B), grown for 12 hrs, and treated for 24 hrs with the indicated concentrations of LE extracts along with 1 μg/ml LPS. Luciferase activities were determined as described in Materials and Methods. Data shown are the mean values ± SD (n = 3). *P < 0.05 or **P < 0.01 compared to **compared to control cells treated with LPS alone.**

Inhibition of LPS-induced p38 and JNK signaling pathways by treatment with LE extracts

It has been reported that stimulation of RAW264.7 macrophage cells with LPS resulted in the activation of all three MAPKs, including ERK, JNK, and p38 MAPK (22). The transcription factor AP-1 is one of the downstream targets of ERK and JNK (23), while the p38 MAPK is required for NF-κB-dependent gene expression (24). Treatment of cells with LE extracts inhibited the LPS-induced phosphorylation of JNK and p38 MAPK, whereas it had no inhibitory effect on the phosphorylation of ERK in response to LPS (Fig. 4C). Inhibition of the downstream JNK signal transduction pathway was subsequently confirmed by attenuation of LPS-induced c-Jun phosphorylation (Fig. 4C). The inhibitory effects of LE extracts on the phosphorylation of JNK and p38 protein kinases are comparable to those of specific inhibitors, SP600125 and SB203580, respectively (Fig. 4D). Our results indicate that LE extracts suppress the MAPK pathways upstream of AP-1 and NF-κB, which in turn inhibits AP-1-dependent and/or NF-κB-dependent gene expression in LPS-activated macrophage cells.

DISCUSSION

A large number of medicinal plants have long been used for prevention and treatment of various diseases in Asian countries. Recently, plant remedies have become increasingly popular and are widely used for healthcare and cancer prevention. It

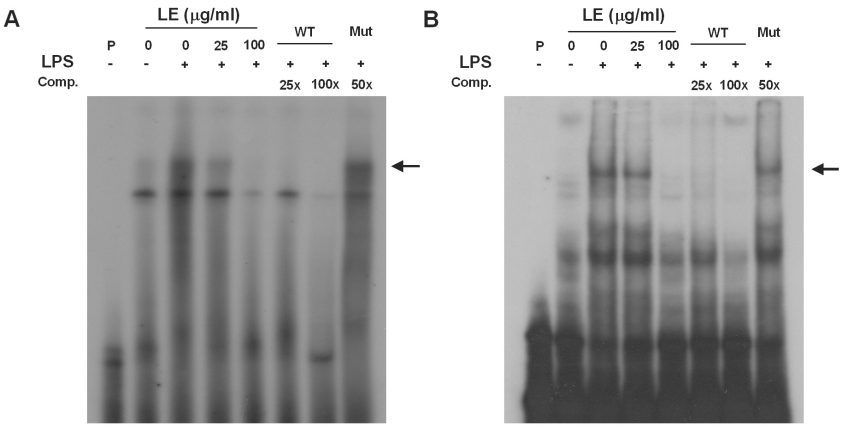

Fig. 3. Effect of LE extracts on LPS-induced DNA binding activity of AP-1 and NF-κB. RAW264.7 cells were treated with LPS (1 μg/ml) in the presence of various concentrations of LE extracts and analyzed for the DNA binding activity to an oglionucleotide containing a consensus motif for AP-1 (A) or NF-κB (B). Nuclear extracts were obtained from RAW 264.7 cells stimulated with LPS for 1 hr. For specific competition, a 25- and 100-fold excess of unlabeled AP-1 or NF-κB consensus oligonucleotides was added to the reaction mixture.

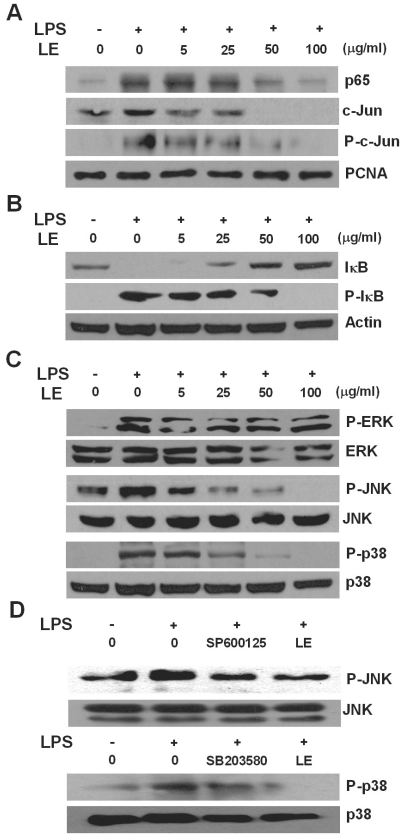

Fig. 4. Effect of the LE extracts on the phosphorlyation and protein levels of IκB, NF-κB, AP-1 and MAP kinases. (A) Nuclear extracts were prepared from RAW264.7 cells untreated or treated with LPS and LE extracts and were analyzed by western blot analysis using anti-c-Jun, anti-phospho-c-Jun, and anti-p65 monoclonal antibodies. The blot was stripped and reprobed with antibody against PCNA to verify equal loading of proteins in each lane. (B) Cytosolic proteins were prepared from RAW264.7 cells untreated or treated with LPS and LE extracts and were analyzed by western blot analysis using anti-IκB-α and anti-phospho-IκB-α polyclonal antibodies. The blot was stripped and reprobed with antibody against actin to verify equal loading of proteins in each lane. (C) RAW264.7 cells were treated with LPS (1 μg/ml) in the presence of various concentrations of LE extracts for 24 hrs. ERK, JNK and p38 MAP kinases were detected by specific antibodies against their corresponding phosphorylated forms. The blots above were then stripped and reprobed with the antibodies raised against the corresponding MAPKs. (D). RAW264.7 cells were treated with LPS in the presence of SP600125 (20 nM) or SB203580 (50 nM), and were analyzed by western blot analysis using JNK and p38 antibodies.

was previously reported that LE extracts inhibited LPS-induced expression of inducible NO synthase and TNF-α in mouse peritoneal macrophages probably via inhibition of NF-κB (17). Therefore, it was necessary to elucidate the molecular mechanisms by which they function. In this study, we showed that extracts from the roots of *Lithospermum erythrorhizon*, which is widely used for the treatment of inflammation and a number of immune disorders in oriental medicine, inhibited LPS-induced expression of pro-inflammatory cytokine genes, including TNF-α, IL-1β, IL-6, and IFN-γ, in macrophage cells.

We investigated the molecular mechanism by which LE extracts inhibit cytokine gene expression, paying particular attention to the role of a transcription factor, AP-1, involved in cytokine production. Our results indicate, for the first time, that the activity of the AP-1 transcription factor was significantly down-regulated by LE extracts in LPS-stimulated macrophage cells. In addition, we also confirmed the inhibitory effects of LE extracts on NF-κB activation using both a luciferase assay system and DNA binding analysis. The inhibition appears to be mediated through the suppression of LPS-activated JNK and p38 MAPK signaling pathways.

AP-1 is a heteodimeric transcription factor that is comprised of members of the Jun and Fos proto-oncogene families (25). It has been implicated in tumor promotion based on the fact that AP-1 factors alter gene expression in response to tumor promoters (12, 13). Inhibition of AP-1 activation by a variety of agents has been shown to reduce neoplastic transformation (13). Various Fos and Jun dimers associate with the promoters of cytokine genes, often in cooperation with other transcription factors, such as NF-κB, NFIL6 and NFAT (25, 26). For example, AP-1 complexes have been shown to interact with NF-κB proteins and synergistically up-regulate expression of TNF-α in LPS-stimulated THP-1 cells (27).

It has also been reported that synergistic activation of cytokine genes by NF-κB and AP-1 plays critical roles in the production of mediators involved in inflammation. It was demonstrated that LE extracts inhibited AP-1-dependent as well as NF-κB-dependent transactivation. EMSA analysis showed a marked increase in the formation of DNA complexes with NF-κB and AP-1 factors after LPS treatment. LE extracts inhibited binding of the transcription factors to their consensus sequences. Thus, inhibition of NF-κB and AP-1-dependent transcription may be a major factor in LE extract-mediated inhibition of cytokine gene expression in stimulated macrophage cells.

AP-1 and NF-κB transcription factors are activated by members of the mitogen-activated protein kinase family that, upon stimulation, enter the nucleus and phosphorylate DNA-bound transcription factors. IκB is phosphorylated and degraded in the cytoplasm, thereby releasing NF-κB and allowing it to translocate to the nucleus and bind NF-κB binding motifs in the regulatory region of a variety of genes (11, 21). This study indicated that LE extracts inhibit the phosphorylation of IκB in LPS-stimulated cells, suggesting that LE extracts block the LPS-induced activation of NFκB by inhibiting the phosphorylation and degradation of IκB. We also demonstrated that the phosphorylation of three MAP kinases, ERK, JNK and p38 MAPK, was highly induced by LPS treatment and that LE extracts significantly inhibited the phosphorylation of JNK and p38 MAPK. However, LE extracts had little effect on the phosphorylation of ERK. These results are consistent with the notion that phosphorylation of the N-terminal sites of c-Jun parallels the activation of

JNK but does not correlate with ERK activation (28).

Given the importance of cytokines in causing inflammation, the inhibition of cytokine production by LE extracts is likely to be clinically relevant. Activation of NF-κB and AP-1 is frequently detected in tumors. The inhibitory effects of LE extracts on NF-κB and AP-1 noted in this study may have a beneficial role in preventing inflammation and carcinogenesis *in vivo*. The present study extends our understanding of the molecular mechanisms underlying the diverse biological activities of LE extracts that are used in traditional medicine. The active ingredient of LE extracts that inhibits angiogenesis and exhibits anti-tumor activities *in vivo* needs to be further investigated.

MATERIALS AND METHODS

Preparation of LE extracts

The roots of *Lithospermum erythrorhizon* were extracted three times with 95% methanol overnight at room temperature. After filtration, the MeOH extract was concentrated using a rotary vacuum evaporator and lyophilized. The extract samples were dissolved in DMSO, aliquoted and stored at -4°C until use.

Cell culture

Mouse RAW 264.7 peritoneal macrophage cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin and streptomycin (Invitrogen) and were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were stimulated with LPS (Sigma) at 1 μg/ml concentrations along with various concentrations of LE extracts for 24 hrs as indicated.

Reverse transcriptase-PCR analysis and real-time PCR

Total RNA was purified from RAW264.7 cells using modified Trizol reagent (Invitrogen). Total RNA (2 μg) was reverse transcribed using M-MuLV reverse transciptase (Fermentas Life Sciecne) and PCR was performed as previously described using specific primers (8). Real time PCRs were performed by a fluorescence detection method using the LightCycler System with a First-Start DNA Master SYBR Green I kit (Roche Diagnostics) as described previously (29). A negative control without cDNA template was performed to assess the overall specificity.

ELISA assays

Mouse RAW 264.7 peritoneal macrophage cells were cultured in 48-well plates for 24 hrs, washed with PBS and treated with various concentrations of LE extracts and LPS for 24 hrs. The levels of cytokines in the supernatant of cultures were measured by the procedures recommended by the supplier (Bender MedSystems).

Transfection and luciferase reporter assays

Transient transfections were carried out using Lipofectamine as described by the manufacturer (Invitrogen). Transfected cells were treated with various concentrations of LE extract and LPS for 24 hrs, harvested and lysed in Report lysis buffer (Promega). Luciferase activities were measured using an enhanced luciferase assay kit (Promega). Experiments were repeated at least three times with duplicate samples, and values are expressed as means ± standard deviations (SD).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from mouse macrophage RAW264.7 cells were prepared as previously described (8). EMSA was performed as described previously. Briefly, double-stranded oligonucleotides containing NF-κB- and AP-1-binding sites were used as probes and specific competitors. Oligomer probes were radiolabelled with [γ-^{32}P] ATP (Amersham) by T4 polynucleotide kinase and purified on sodium Tris-EDTA (STE)-10 columns (BD Biosciences). For competition studies, excess unlabeled specific or mutated oligonucleotides were pre-mixed with the nuclear extract at room temperature for 20 min prior to addition of the labeled probe. The reaction products were separated on 6% polyacrylamide gels. The gels were dried and subjected to autoradiography.

Western blot analysis

RAW 264.7 cells were lysed by adding 200 μl of RIPA lysis buffer (Pierce) containing proteinase inhibitors. Protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories). Rabbit anti-iNOS, anti-p65, anti-c-Fos, anti-c-Jun (Research Santa Cruz), anti-IκB, anti-phospho-IκB (R&D Systems), and anti-actin (Sigma) antibodies were used as primary antibodies and peroxidase-conjugated antibody was used as secondary antibody. Membranes were developed with enhanced chemiluminescent reagents (Amersham) and exposed to X-ray film for 60 seconds.

Statistical analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as means ± SD and statistical comparisons between groups were performed using 1-way ANOVA followed by Student's *t*-test.

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