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Mechanisms Underlying *Enterococcus faecalis*-Induced Tumor Necrosis Factor-α Production in Macrophages

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***Enterococcus faecalis*, a gram-positive bacterium, has been implicated in endodontic infections, particularly in chronic apical periodontitis. Proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), are involved in the pathogenesis of these apical lesions. *E. faecalis* has been reported to stimulate macrophages to produce TNF-α. The present study investigated the mechanisms involved in TNF-α production by a murine macrophage cell line, RAW 264.7 in response to exposure to *E. faecalis*. Both live and heat-killed *E. faecalis* induced high levels of gene expression and protein release of TNF-α. Treatment of RAW 264.7 cells with cytochalasin D, an inhibitor of endocytosis, prevented the mRNA up-regulation of TNF-α by *E. faecalis*. In addition, antioxidant treatment reduced TNF-α production to baseline levels. Inhibition of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase also significantly attenuated *E. faecalis*-induced TNF-α expression by RAW 264.7 cells. Furthermore, activation of NF-κB and AP-1 in RAW 264.7 cells was also stimulated by *E. faecalis*. These results suggest that the phagocytic uptake of bacteria is necessary for the induction of TNF-α in *E. faecalis*-stimulated macrophages, and that the underlying intracellular signaling pathways involve reactive oxygen species, ERK, p38 MAP kinase, NF-κB, and AP-1.**

Key words: *Enterococcus faecalis*, tumor necrosis factor-α, macrophage

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Introduction

Periapical lesions of pulpal origin develop in response to microbial irritants in the root canal systems (Stashenko *et al*., 1998). *Enterococcus faecalis*, a gram-positive facultative bacterium, has been implicated in endodontic infections (Stuart *et al*., 2006). It has been frequently found in obturated root canals exhibiting sings of chronic apical periodontitis, isolated in 23-70% of the positive cultures and often occur in monoculture (Hancock *et al*., 2001). Moreover, *E. faecalis* was among a group of bacteria cultured from periapical lesions refractory to endodontic treatment (Sunde *et al*., 2002).

Apical periodontitis is viewed as the consequence of a dynamic encounter between root canal microbes and host defense. Bacteria and their cell wall components react with monocytes/macrophages and other cells of the immune system and fibrobalsts, leading to the production of proinflammatory cytokines, such as IL-1β and TNF-α. The presence of TNF-α has been reported in human apical periodontitis lesions and root canal exudates of teeth with apical periodontitis (Stuart *et al*., 2006). This proinflammatory cytokine has manifold effects on tissue cells, for example, stimulation of osteoclastic bone resorption. It also increases the local vascular response and stimulates lymphocytes (Danin *et al*., 2000).

Multiple signaling mechanisms have been reported to be involved in the intracellular activation of TNF-α gene expression in macrophages by various stimuli. A major mechanism through which signals from environmental stimuli are transmitted to the nucleus involves the activation of cellular kinases, including those belonging to the mitogen-activated

protein (MAP) kinase superfamily (Robinson and Cobb, 1997). The MAP kinase group consists of three serine-threonine kinases: extracellular signal-regulated kinases (ERK) and the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 MAP kinase (Kim *et al*., 2005). Reactive oxygen species (ROS) are now considered as signaling molecules that are generated in response to proinflammatory stimuli (Forman *et al*., 2002). The promoter region of TNF-α gene contains binding sites for the redox-responsive transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), which have been shown to be important for TNF-α expression. Cytokines are selectively expressed in a cell type-specific and stimulus-specific manner and the clarification of differential activation of intracellular signaling pathways provides a critical understanding for therapeutic intervention.

Recently, Baik *et al.* reported that *E. faecalis* and its lipoteichoic acid (LTA) strongly induce TNF-α expression in macrophages (Baik *et al*., 2008a; Baik *et al*., 2008b). However, the cellular mechanisms mediating this TNF-α production have been largely unknown. The present study was undertaken to elucidate the signaling mechanisms involved in the TNF-α induction by *E. faecalis* in macrophages.

Matrials and Methods

Reagents

GF109203X, U73122, PD98059, SB203580, and SP600125 were purchased from Calbiochem (USA). Wortmannin and genistein were purchased from Sigma (USA). Antibodies to phospho-IκB kinase, IκB-α, phospho-ERK, phospho-p38, and phosphor-JNK were from Cell Signaling Technology (USA). Anti-β-actin was from Sigma. Unless stated otherwise, all chemicals were from Sigma. Highly pure *E. faecalis* LTA was kindly provided by Dr. Seung Hyun Han (Department of Oral Microbiology & Immunology, School of Dentistry, Seoul National University).

Bacteria and growth condition

E. faecalis ATCC 29212 was purchased from the American Type Culture Collection (ATCC). It was grown anaerobically (85% N_2, 10% H_2, and 5% CO_2) in brain heart infusion broth at 37°C. Bacteria in logarithmic growth phase were used in all experiments. For the preparation of heat-killed *E. faecalis*, bacteria were harvested, washed three times with phosphate-buffered saline (PBS), and resuspended in a small volume of PBS. Then the bacteria were exposed to heat (80°C for 30 min).

Cell culture

The mouse macrophage cell line, RAW 264.7, was purchased from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 50 μg/ml gentamicin (GM) at 37°C in a humidified incubator with 5% CO_2.

Infection protocol

Bacterial were washed twice with PBS and once with DMEM medium. After resuspension in DMEM medium, the optical density of the bacterial suspension was measured at 600 nm, and further diluted to an optical density of 0.5 which corresponded to 5×10^8 CFU/ml. The live or killed bacterial suspensions of OD of 0.5 were further diluted with complete DMEM medium including GM as needed. Even in the presence of GM, growth of live *E. faecalis* was occurred. For enzyme-linked immunosorbent assays (ELISA), 5×10^5 RAW 264.7 cells were seeded in 24-well plates. For Western blots, RT-PCR, and gel shift assays, 1×10^6 cells were seeded in 3-cm culture dishes. The next day, the medium was removed and the cells were incubated with various doses of live or heat-killed *E. faecalis* for various times.

Measurement of TNF-α from culture supernatants

The culture supernatants of RAW 264.7 cells were collected, clarified, and the levels of TNF-α were quantified using a commercial ELISA kit (R&D Systems, USA) according to the manufacturer's directions.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared with Trizol reagent (Invitrogen) as specified by the manufacturer and was quantified spectrophotometrically. First-strand cDNA was synthesized from 1 μg of RNA using random primers (Promega, USA) and Molony murine leukemia virus reverse transcriptase (Invitrogen). 2 μl of cDNA products was amplified in 25 μl volumes under a layer of mineral oil using a GeneAmp 2700 thermal cycler (Applied Biosystems, USA) for 25 cycles. Each PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 U *Taq* DNA polymerase, and 0.5 μM of each primer. Each cycle consisted of denaturation at 94°C (30 s), annealing at 60°C (30 s), and extension at 72°C (60 s). All of the PCR reagents were from Promega. The sequences of primers were 5'-GTGACAAGCCTGTAGCCCA-3', 5'-AAAGTAGACCTGCCCGGAC-3' for TNF-α (428 bp) and 5'-GATCTGGCACCACACCTTCT-3', 5'-GGGGTGTTGAAGGTCTCAAA-3' for β-actin (132 bp). The PCR products of 10 μl were fractionated on 1.2% agarose gels containing ethidium bromide, visualized by UV transillumination, and photographed.

Western blot

RAW 264.7 cells were scraped and lysed with 100 μl of Cell Lysis Buffer (Cell Signaling Technology, USA). 40 μg of each boiled sample was resolved by SDS-PAGE (10%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). The membrane was probed with a 1:1000 dilution of rabbit anti-phospho-IKK polyclonal antibody and a 1:1500 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology). Immunoreactive proteins were detected by enhanced

chemiluminescence (LumiGLO, Cell Signaling Technology). The same membrane was successively stripped and reprobed with anti-IκB-α (1:1000), anti-phospho-ERK (1:1000), anti-phospho-p38 (1:1000), anti-phospho-JNK (1:1000), and anti-β-actin (1:5000).

Nuclear extract preparation and gel shift assays

For the preparation of nuclear extracts, cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9 at 4°C, 0.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF) and incubated for 10 min on ice, then the cells were lysed by addition of 10% IGEPAL CA-630, followed by vigorous vortex for 10 s. Nuclei were pelleted and resuspended in low-salt buffer (20 mM HEPES, pH 7.9 at 4°C, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and added high-salt buffer (20 mM HEPES, pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 0.8 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) in a drop-wise fashion. After 30 min incubation at 4°C, the lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. Protein concentrations of the nuclear extracts were measured with DC Protein Assay Kit (Bio-Rad). Double-stranded NF-κB-binding DNA probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') and AP-1-binding DNA probe (5'-CGCTTGATGAGTCAGCCGGAA-3') were purchased from Promega, and end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. About 10 μg of nuclear extracts were incubated with 10,000 cpm of probe in 20 μl of reaction buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, and 200 ng of poly(dI·dC) for 30 min at room temperature. The DNA-protein complexes were separated on 4% polyacrylamide gels. The gels were dried and subjected to autoradiography.

Results

Dose- and time-dependent production of TNF-α by *E. faecalis*

RAW264.7 cells were incubated with medium alone or increasing doses of live or killed *E. faecalis* for 12 h, and TNF-α concentrations of the collected supernatants were measured by ELISA. Increased concentrations of TNF-α were obtained with increasing inoculums of bacteria up to a multiplicity of infection (MOI) of 1:500. Both live and killed *E. faecalis* increased the production of TNF-α to a similar extent. A 10-fold increase of TNF-α production was observed following an MOI as low as 1:1. A robust response of more than a 20-fold increase of TNF-α production was demonstrated with an MOI of 1:10 (Fig. 1a). Heat-killed *E. faecalis* at an MOI of 1:10 was used for other later experiments. Next, in order to examine the time course of the TNF-α release, RAW 264.7 cells were challenged with killed *E. faecalis* for up to 36 h. The amount of TNF-α release was significantly enhanced after 3 h of stimulation and continued to increase up to 24 h, after which the TNF-α levels were slightly decreased (Fig. 1b).

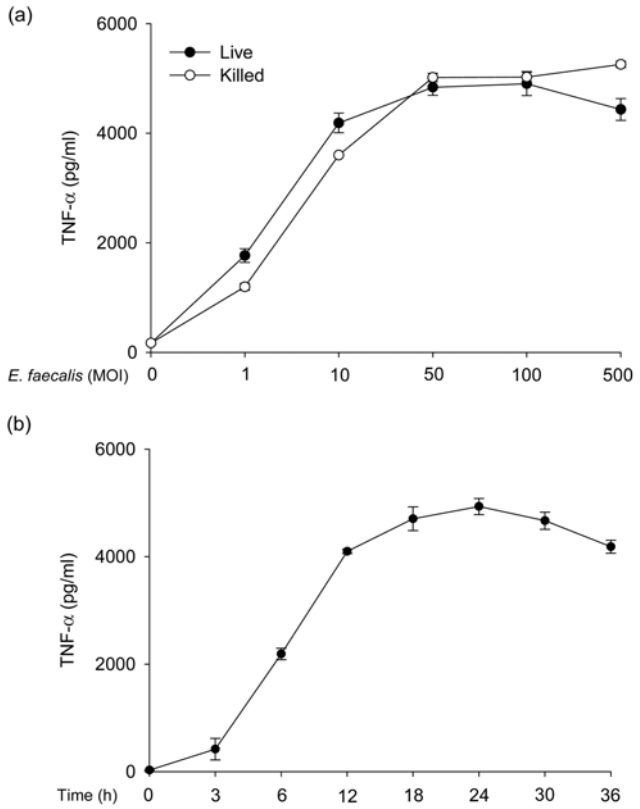

Fig. 1. Dose- and time-dependent production of TNF-α by *E. faecalis* in RAW 264.7 cells. 5×10^5 RAW 264.7 cells were seeded in 24-well plates. The next day, the cells were incubated with different MOIs of live or heat-killed *E. faecalis* in a final volume of 1 ml for 12 h. TNF-α concentrations of the culture supernatants were measured by ELISA (a). RAW 264.7 cells were challenged with heat-killed *E. faecalis* (MOI = 1:10) for up to 36 h (b). Values are means ± SD of triplicate samples.

Production of TNF-α by *E. faecalis* LTA

LTA is a well known cytokine-inducing constituent of gram-positive bacteria and *E. faecalis* LTA increases TNF-α production in macrophages. It was of interest to compare the magnitude of TNF-α production by *E. faecalis* LTA and *E. faecalis* whole cells. RAW264.7 cells were incubated with various doses (1-30 μg/ml) of *E. faecalis* LTA for 12 h, and TNF-α level of the collected supernatants were measured by ELISA. *E. faecalis* LTA stimulated RAW 264.7 cells to produce TNF-α in a concentration-dependent manner. A 10-fold increase of TNF-α production was observed only with a relatively high concentration of 30 μg/ml (Fig. 2)

Induction of TNF-α mRNA expression by *E. faecalis*

In order to examine the induction of TNF-α at the steady-state mRNA level, RT-PCR analysis was performed on RNA extracted from RAW 264.7 cells that had been stimulated by

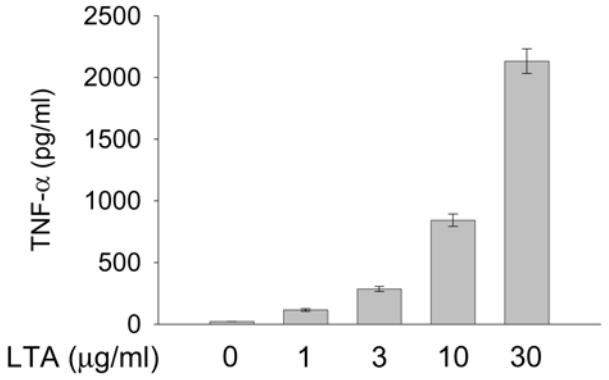

Fig. 2. Production of TNF-α by *E. faecalis* LTA in RAW 264.7 cells. 5×10^5 RAW 264.7 cells were seeded in 24-well plates. The next day, the cells were stimulated with different doses of *E. faecalis* LTA in a final volume of 1 ml for 12 h. TNF-α concentrations of the culture supernatants were measured by ELISA. Values are means ± SD of triplicate samples.

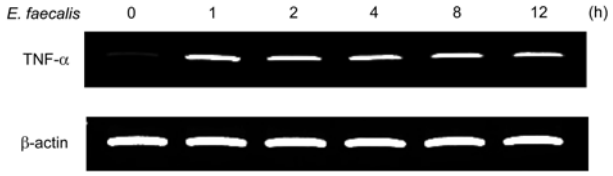

Fig. 3. Induction of TNF-α mRNA expression by *E. faecalis* in RAW 264.7 cells. 1×10^6 RAW 264.7 cells were seeded in 3-cm culture dishes. The next day, the cells were incubated with heat-killed *E. faecalis* at an MOI of 1:10 in a final volume of 2 ml. Total RNA was isolated at 1, 2, 4, 8, and 12 h after stimulation, and levels of TNF-α mRNA were determined by RT-PCR.

E. faecalis for 1, 2, 4, 8 and 12 h. The TNF-α mRNA expression in RAW 264.7 cells was increased as early as 1 h after *E. faecalis* stimulation and maintained throughout the 12-h time period (Fig. 3).

Activation of MAP kinases by *E. faecalis*

As the MAP kinases are central to many inflammatory signaling pathways, it was determined whether MAP kinases are activated by *E. faecalis* in RAW 264.7 cells. Western blot analysis was performed for phospho-p38, -ERK, or -JNK. Activation of MAP kinases occurs through phosphorylation of threonine and tyrosine residues by upstream MAP kinase kinases. Exposure of RAW 264.7 cells to *E. faecalis* for 30-60 min brought about phosphorylation of all three members of MAP kinases. Upregulation of the phosphorylation of ERK and p38 was continued up to 4 h after stimulation (Fig. 4).

Activation of IKK by *E. faecalis*

IKK activation and subsequent IκB degradation are the canonical pathway for NF-κB activation in response to a given stimulus. To determine whether *E. faecalis* stimulates this pathway, RAW 264.7 cells were challenged with *E. faecalis*, and Western blot analysis was performed for phospho-IKK and IκB-α. Phosphorylation of IKK and degradation of

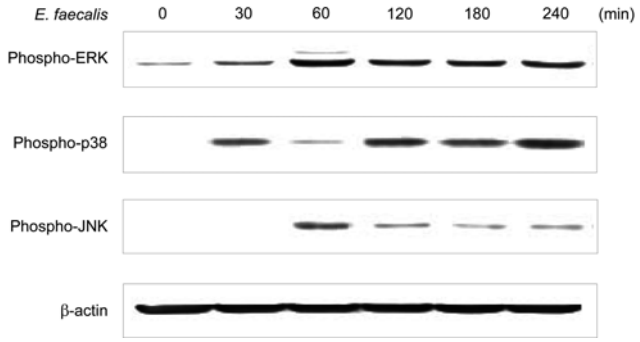

Fig. 4. Activation of MAP kinases by *E. faecalis* in RAW 264.7 cells. 1×10^6 RAW 264.7 cells were seeded in 3-cm culture dishes. The next day, the cells were incubated with heat-killed *E. faecalis* at an MOI of 1:10 for the indicated time periods in a final volume of 2 ml. Cell lysates were prepared and Western blot analysis was performed for phospho-ERK, phospho-p38, or phospho-JNK.

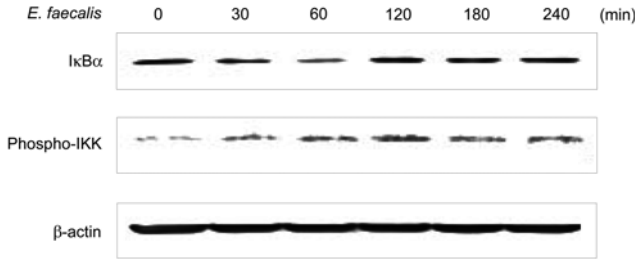

Fig. 5. Activation of IKK by *E. faecalis* in RAW 264.7 cells. 1×10^6 RAW 264.7 cells were seeded in 3-cm culture dishes. The next day, the cells were incubated with heat-killed *E. faecalis* at an MOI of 1:10 for the indicated time periods in a final volume of 2 ml. Cell lysates were prepared and Western blot analysis was performed for phospho-IKK or IκBα.

IκB-α by *E. faecalis* were demonstrated, in which IKK phosphorylation began at 30 min and degradation of IκB-α was most at 1 h after stimulation (Fig. 5).

Activation of transcription factors NF-κB and AP-1 by *E. faecalis*

Since NF-κB and AP-1 are critical transcription factors for TNF-α gene expression, activation of NF-κB and AP-1 by *E. faecalis* was examined. The DNA-binding activity of AP-1 or NF-κB in *E. faecalis*-exposed RAW 264.7 cells was analyzed by gel shift assays. RAW 264.7 cells were stimulated with *E. faecalis* for 30 or 60 min. As shown in Fig. 6, strong inducible NF-κB and AP-1 DNA binding activities were detected after stimulation with *E. faecalis*.

Role of bacterial internalization

It was of interest to determine whether internalization of bacteria is necessary or adhesion itself is sufficient for TNF-α induction by *E. faecalis* in RAW 264.7 cells. For this purpose, RAW 264.7 cells were pretreated for 30 min with cytochalasin D (1 μg/ml), followed by *E. faecalis* stimulation for 8 h. Cytochalasin D, acting as an inhibitor of actin polymerization, prevents uptake of bacteria without interfering

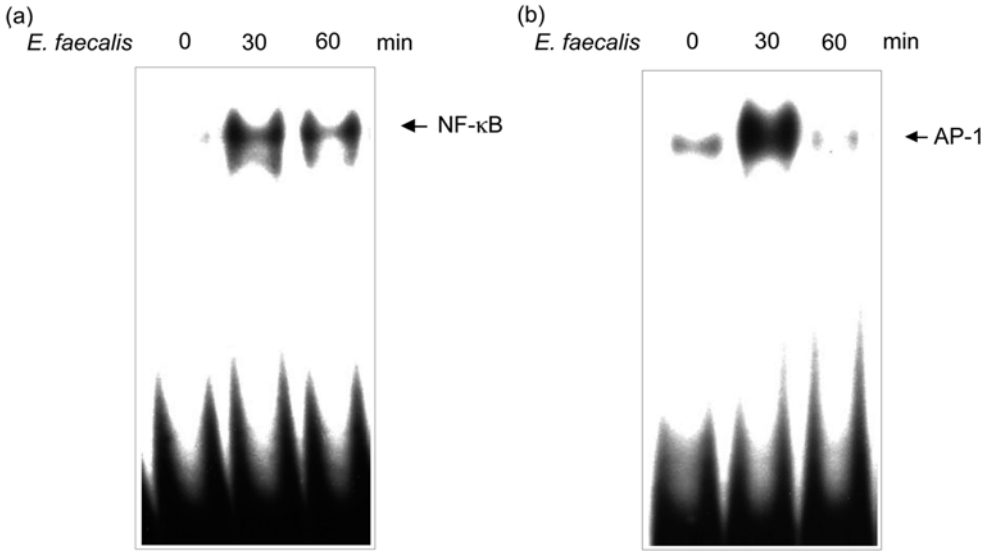

Fig. 6. Activation of transcription factors NF-κB and AP-1 by *E. faecalis*. 1×10^6 RAW 264.7 cells were seeded in 3-cm culture dishes. The next day, the cells were incubated with heat-killed *E. faecalis* at an MOI of 1:10 for 30 or 60 min in a final volume of 2 ml. Nuclear extracts were prepared and the DNA binding activity of NF-κB (a) and AP-1 (b) was determined by gel shift assays.

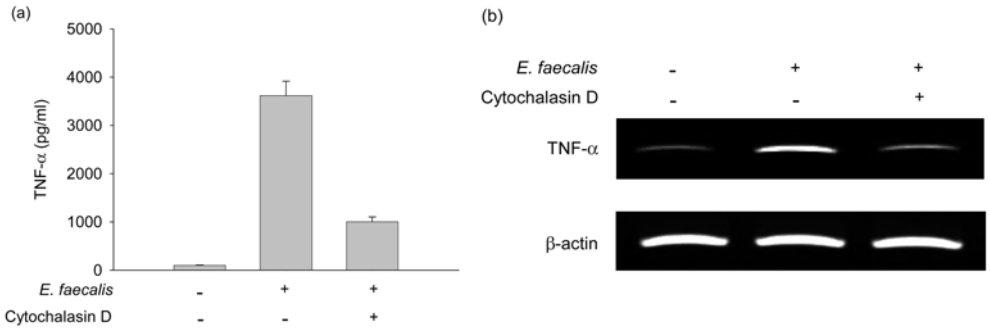

Fig. 7. Effect of cytochalasin D on TNF-α production by *E. faecalis* in RAW 264.7 cells. (a) 5×10^5 RAW 264.7 cells were seeded in 24-well plates. The next day, the cells were pretreated for 30 min with cytochalasin D (1 μg/ml), and then incubated with heat-killed *E. faecalis* in a final volume of 1 ml for 8 h. TNF-α concentrations of the culture supernatants were measured by ELISA. Values are means ± SD of triplicate samples. (b) 1×10^6 RAW 264.7 cells were seeded in 3-cm culture dishes. The next day, the cells were pretreated for 30 min with cytochalasin D, and then incubated with heat-killed *E. faecalis* in a final volume of 2 ml. Total RNA was isolated at 1 h after stimulation, and levels of TNF-α mRNA were determined by RT-PCR.

with the binding of bacteria. Cytochalasin D treatment of RAW 264.7 cells prevented TNF-α production by *E. faecalis*, which was demonstrated at both protein and mRNA levels (Fig. 7).

Involvement of ROS in *E. faecalis*-induced TNF-α expression

An emerging body of evidence supports a role for ROS in intracellular signaling. To determine whether the *E. faecalis*-induced TNF-α production is mediated by an ROS-sensitive mechanism, RAW 264.7 cells were pretreated for 30 min with *N*-acetyl-L-cysteine (NAC, 10 mM), followed by *E. faecalis* stimulation for 8 h. NAC is a well-characterized thiol-containing antioxidant. As shown in Fig. 8, NAC significantly inhibited the TNF-α production. Moreover, TNF-α mRNA expression was also reduced by NAC.

Involvement of ERK and p38 MAP kinase in *E. faecalis*-induced TNF-α production

In order to evaluate the relative importance of MAP kinases and some additional signaling pathways in *E. faecalis*-induced TNF-α production in endothelial cells, specific pharmacological inhibitors were used. RAW 264.7 cells were pretreated with GF109203X (protein kinase C, 1 μM), wortmannin (phosphatidylinositol 3-kinase, 100 nM), U73122 (phospholipase C, 10 μM), PD98059 (ERK, 50 μM), SB-203580 (p38 MAP kinase, 10 μM), or SP600125 (JNK, 10 μM) for 30 min, and then the cells were incubated with *E. faecalis* for 8 h. TNF-α concentrations of the collected supernatants were measured by ELISA. Among these inhibitors, PD98059 and SB203580 reduced TNF-α level most strongly, which was also demonstrated at the mRNA level (Fig. 9).

Discussion

Bacteria and the inflammatory reactions induced by their metabolic products, enzymes, and toxins are the main cause of periapical diseases. Among microorganisms identified as peculiar to endodontic infections, *E. faecalis* has gained considerable attention by its frequent recovery in root-filled teeth with apical periodontitis after endodontic treatment (Hancock *et al*., 2001; Stuart *et al*., 2006; Sunde *et al*., 2002). *E. faecalis* can colonize the root canals and survive even in harsh conditions. During the acute phase of apical periodontitis, macrophages appear at the periapex. Activated macrophages produce a variety of mediators, among which the proinflammatory (TNF-α and IL-1) and chemotactic (IL-8) cytokines are of particular importance. TNF-α intensifies the local vascular response, osteoclastic bone resorption, and effector-mediated degradation of the extracellular matrices (Danin *et al*., 2000; Stuart *et al*., 2006).

In this study, *E. faecalis* strongly induced the production of TNF-α in murine macrophagic RAW 264.7 cells. There was no difference between live and killed *E. faecalis* in terms of their ability to stimulate RAW 264.7 cells to produce TNF-α. Killed bacteria were used in most experiments in this study because killed *E. faecalis* preparation was more convenient to use for a series of experiments. LTA is one of the major virulence factors of *E. faecalis* and LTA triggers inflammatory responses. In this study, although *E. faecalis* LTA induced the production of TNF-α, the amount of TNF-α was smaller compared to *E. faecalis* whole cell-stimulated TNF-α production. Moreover, the amount of LTA present in *E. faecalis* used in the *in vitro* and *in vivo* assays should be much lower than the amount used with purified LTA. These suggest that the strong TNF-α production in response to *E. faecalis* is not solely due to its LTA.

This study demonstrated the importance of p38 MAP kinase and ERK in *E. faecalis*-stimulated TNF-α production by RAW 264.7 cells. Increased phosphorylation of ERK and p38 was observed in *E. faecalis*-stimulated RAW 264.7 cells. Pharmacological inhibition of p38 or ERK substantially reduced the TNF-α expression. Indeed, ERK and p38 are required for TNF-α expression induced by LPS in cardiomyocytes. Transcription of TNF-α gene requires binding of transcription factors, including NF-κB and AP-1, to the

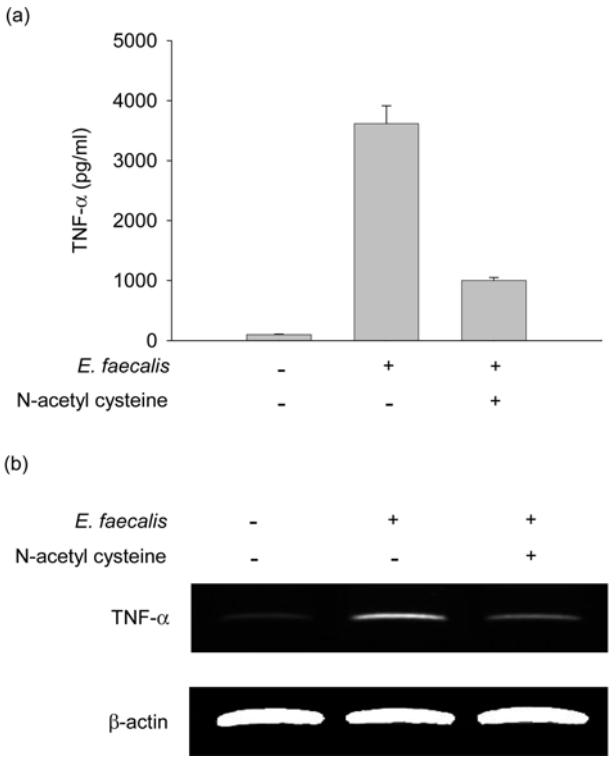

Fig. 8. Effect of NAC on TNF-α production by *E. faecalis* in RAW 264.7 cells. (a) 5×10^5 RAW 264.7 cells were seeded in 24-well plates. The next day, the cells were pretreated for 30 min with NAC (10 mM), and then incubated with heat-killed *E. faecalis* in a final volume of 1 ml for 8 h. TNF-α concentrations of the culture supernatants were measured by ELISA. Values are means ± SD of triplicate samples. (b) 1×10^6 RAW 264.7 cells were seeded in 3-cm culture dishes. The next day, the cells were pretreated for 30 min with NAC, and then incubated with heat-killed *E. faecalis* in a final volume of 2 ml. Total RNA was isolated at 1 h after stimulation, and levels of TNF-α mRNA were determined by RT-PCR.

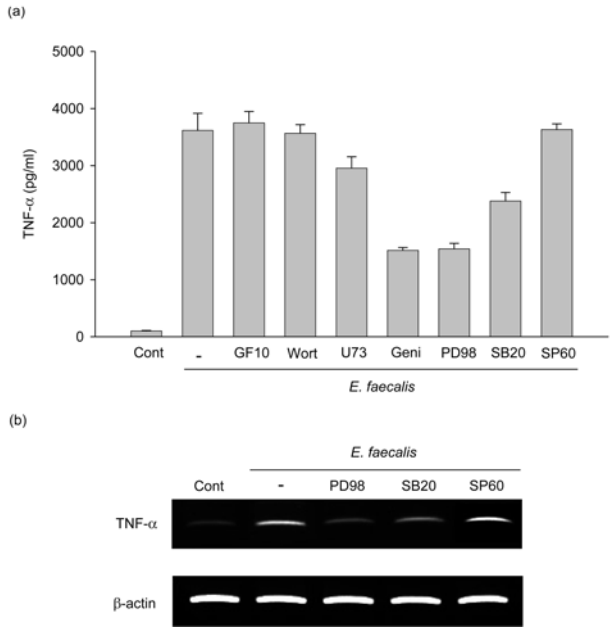

Fig. 9. Effect of various signaling inhibitors on TNF-α production by *E. faecalis* in RAW 264.7 cells. (a) 5×10^5 RAW 264.7 cells were seeded in 24-well plates. The next day, the cells were pretreated for 30 min with GF109203X (1 μM), wortmannin (100 nM), U73122 (10 μM), PD98059 (50 μM), SB203580 (10 μM), or SP600125 (10 μM), and then incubated with heat-killed *E. faecalis* in a final volume of 1 ml for 8 h. TNF-α concentrations of the culture supernatants were measured by ELISA. Values are means ± SD of triplicate samples. (b) 1×10^6 RAW 264.7 cells were seeded in 3-cm culture dishes. The next day, the cells were pretreated for 30 min with PD98059, SB203580, or SP600125, and then incubated with heat-killed *E. faecalis* in a final volume of 2 ml. Total RNA was isolated at 1 h after stimulation, and levels of TNF-α mRNA were determined by RT-PCR.

promoter region of TNF-α gene. As MAP kinases regulate these transcription factors, one possible role for upregulated p38 and ERK in *E. faecalis*-induced TNF-α expression is the positive regulation of the activity of transcription factors. The canonical pathway for activation of NF-κB consists of IKK activation and subsequent degradation of IκB. Removal of IκB from NF-κB allows NF-κB to translocate into the nucleus (Baeuerle and Henkel, 1994). AP-1 factor is composed of the proteins Fos and Jun. Activation of AP-1 typically involves synthesis of the Fos protein and phosphorylation of preexisting Jun protein. Transcription and synthesis of Fos can be enhanced by the ERK pathway. JNK phosphorylates c-Jun, and AP-1 complexes containing the phosphorylated form of Jun have increased transcriptional enhancing activity (Robinson and Cobb, 1997). In this study, activation of NF-κB and AP-1 was observed in *E. faecalis*-stimulated RAW 264.7 cells. These results indicate that *E. faecalis* activates both NF-κB and AP-1 in macrophages and the combinational action of the two transcription factors results in enhanced transcription of TNF-α gene.

Cytochalasin D treatment of RAW 264.7 cells prevented TNF-α induction by *E. faecalis*, indicating that TNF-α induction requires internalization of *E. faecalis*. ROS including superoxide anion, hydrogen peroxide, hydroxyl radical are now considered as signaling molecules and ROS are required for LPS-induced TNF-α gene expression in neutrophils. In the present study, TNF-α induction by *E. faecalis* was prevented by antioxidant treatment at both protein and mRNA levels, suggesting the involvement of ROS. Therefore, ROS generation following *E. faecalis* internalization should be an early event that is essential to the TNF-α induction.

E. faecalis are frequently found in endodontic infections. Interactions of macrophages with *E. faecalis* should take place in pulpal and periapical tissues. As a result of interactions of *E. faecalis* with macrophages, many inflammatory cytokines including TNF-α should be released into the local environment, which contributes to the pathogenesis of apical periodontitis. The present study proposes the following signaling pathways involving in *E. faecalis*-induced TNF-α production by macrophages. First, macrophages generate ROS in response to *E. faecalis* internalization, in which phagocytic NADPH oxidase may be the major source of ROS. Second, activation of IKK, ERK, and p38 MAP kinase is followed by a redox-dependent mechanism. Finally, nuclear translocation of NF-κB and activation of AP-1 are achieved, which cooperatively upregulates expression of TNF-α.

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