Enterococcus faecalis Activates Caspase-1 Leading to Increased Interleukin-1 Beta Secretion in Macrophages

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Abstract

Introduction: Recent studies of inflammasome activation have focused on the pathogenesis of diverse inflammatory and autoimmune diseases. Inflammasome activation results in caspase-1 activation, which is required for processing of proinflammatory (IL)-1 beta to its secreted form as well as a proinflammatory cell death (ie, pyroptosis). The purpose of this study was to analyze whether Enterococcus faecalis associated with endodontic infection induces inflammasome activation.

Methods: THP-1 macrophages were treated with E. faecalis in the presence or absence of caspase-1 inhibitors. Caspase-1 activation, pro–IL-1 beta expression, and IL-1 beta secretion were detected by immunoblotting, real-time reverse-transcription polymerase chain reaction, and enzyme-linked immunosorbent assay, respectively. Cell death was measured by lactate dehydrogenase release and propidium iodide staining. Adenosine triphosphate (ATP) release was measured by an ATP bioluminescence assay kit.

Results: E. faecalis induced caspase-1 activation and pro–IL-1 beta expression, which resulted in IL-1 beta secretion in macrophages. E. faecalis significantly induced ATP release, which is a mechanism of Nod-like receptor family protein 3 (NLRP3) inflammasome activation, whereas oxATP treatment inhibited E. faecalis–induced caspase-1 activation. E. faecalis significantly increased lactate dehydrogenase release and propidium iodide uptake, which are characteristics of pyroptosis.

Conclusions: Our results show that E. faecalis may contribute to the progression of pulpal inflammation by stimulating excessive secretion of IL-1 beta and cell death. (J Endod 2014;40:1587–1592)

Key Words

Caspase-1, Enterococcus faecalis, inflammasome, interleukin-1 beta processing, pyroptosis

Enterococcus faecalis is a facultatively anaerobic Gram-positive bacterium. Although E. faecalis is a commensal bacterium that inhabits the gastrointestinal tract and oral cavity, its resistance to multiple antibiotics can result in nosocomial infections (1–3). E. faecalis is a prominent microorganism found in biofilms, which protect bacteria against antibiotics and phagocytosis (4, 5). In particular, E. faecalis is involved in biofilm-mediated infections caused by indwelling medical devices, orthopedic implants, and catheters (6–8). E. faecalis expresses multiple genes involved in biofilm formation and quorum sensing (9–11). E. faecalis is found in secondary and persistent endodontic infections rather than primary endodontic infections and is 1 of the frequently found species in root canal–treated teeth when analyzed by culture-dependent and culture-independent molecular methods (12–18). This bacterium is highly resistant to various irrigants and medicaments that are used for endodontic treatment, including sodium hypochlorite and calcium hydroxide (19–21).

Recent studies on inflammasome activation by bacteria have focused on chronic inflammatory and autoimmune diseases (22). Inflammasome is a multimeric protein complex composed of a cytoplasmic inflammasome sensor molecule, the adaptor protein ASC (apoptosis-associated specklike protein containing a caspase recruitment domain), and procaspase-1. Inflammasome activation results in the maturation of procaspase-1, which, in turn, is required for the maturation of interleukin (IL)-1 beta and proinflammatory cell death (23). IL-1 beta is produced in a proform (31 kDa) in cells and is processed peptidolytically to a mature form (17 kDa). This process is essential for its secretion and functional role. Procaspase-1 in inflammasome complexes is processed to active caspase-1, which comprises tetramers composed of 2 p10 and 2 p20 subunits. IL-1 beta is a key inflammatory cytokine involved in host defense against pathogens. However, excessive secretion of IL-1 beta enhances inflammation, finally leading to tissue damage. Active caspase-1 is also involved in proinflammatory cell death or pyroptosis (24). Although numerous bacteria have been shown to activate caspase-1 in different inflammasome complexes (25), the ability of E. faecalis to activate caspase-1 has not been investigated.

In this study, we analyzed caspase-1 activation by E. faecalis in macrophages to determine if E. faecalis stimulates IL-1 beta processing and pyroptosis as part of the innate immune response.

Materials and Methods

Bacterial Strains and Growth Conditions

E. faecalis (American Type Culture Collection [ATCC] 29212) was cultured in anaerobic atmosphere (10% CO₂, 5% H₂, and 85% N₂) using brain-heart infusion broth (BD Bioscience, San Jose, CA).

Treatment of THP-1 Cells with E. faecalis

E. faecalis was cultured overnight, inoculated to an initial optical density at 600 nm of 0.05 in fresh medium, and cultured for 12 hours. Bacteria in the early stationary phase were harvested by centrifugation at 930g for 20 minutes and washed with phosphate buffered saline (PBS). Bacteria were resuspended in RPMI 1640 medium (HyClone, Logan, UT), and the number of bacteria was determined by counting using a hemocytometer. THP-1 cells (ATCC TIB-202), a human...
monocytic cell line, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin. The cells were seeded in 6-well plates (2 × 10^6 cells/well) and differentiated into macrophage-like cells by treatment with 0.5 μmol/L phospholipid 13:acetate for 3 hours. Phospholipid 12-myristate 13:acetate–differentiated THP-1 macrophages were washed with serum-free medium without antibiotics and treated with E. faecalis at a multiplicity of infection (MOI, the ratio of the number of bacteria to the number of host cells) of 1, 5, 20, and 50 for 6 hours at 37°C in the presence of 5% CO₂.

In some experiments, THP-1 macrophages were pretreated with caspase-1 inhibitors Z-VAAD-FMK (Bio Vision, Palo Alto, CA) and Ac-VAAD-CHO (Calbiochem, San Diego, CA) or oxATP (Sigma-Aldrich, St Louis, MO) for 30 minutes before stimulation with E. faecalis. As a positive control for caspase-1 activation, we used Pam3CSK₄ (InvivoGen, San Diego, CA), a synthetic bacterial lipopeptide.

**Immunoblotting**

Caspase-1 and IL-1 beta in the culture supernatants and pro-caspase-1, pro-IL-1 beta, and beta-actin in the cell lysates were detected by immunoblotting. Cell lysates and supernatants of THP-1 macrophages treated with E. faecalis or Pam3CSK₄ were prepared as follows. After centrifugation of the cells at 600g for 10 minutes, supernatants and cell pellets were collected separately. Supernatants were mixed with 10% trichloroacetic acid at 4°C for 30 minutes and centrifuged at 16,000g for 10 minutes. After removing the trichloroacetic acid, the precipitated pellets were mixed with 15 μL 0.1 N NaOH. Cell pellets were prepared by lysing with RIPA buffer, which is composed of 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X-100 (MERCK, Darmstadt, Germany), 50 mM NaF, 1 mM ethylenediaminetetraacetic acid, 5 μM Na3VO4, 1 mM phenylmethanesulfonflouride, complete protease inhibitor (Roche, Mannheim, Germany). Cell pellets and the precipitated supernatants were separately mixed with 5× sample buffer for SDS-PAGE, subjected to SDS-PAGE sodium dodecyl sulfate- polyacrylamid gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skim milk for 1 hour and reacted with anti-human caspase-1 Ab (recognizes pro-caspase-1 p45 and caspase-1 p20; Cell Signaling, Beverly, MA) in 5% bovine serum albumin at 4°C for 2 days. After washing with PBS-0.1% Tween 20 (AMRESCO, Solon, OH), membranes were reacted with horseradish peroxidase–conjugated goat antirabbit immunoglobulin G (R&D Systems, Minneapolis, MN) in 5% skim milk for 1 hour at room temperature. After washing with PBS-0.1% Tween 20, antibody binding was detected with standard enhanced chemiluminescence reagents. To detect pro–IL-1 beta, IL-1 beta, and beta-actin, membranes were stripped with a stripping solution (62.5 mmol/L Tris-HCl, pH = 6.8, 2% SDS, 0.8% beta-mercaptoethanol) and reacted with the corresponding antibodies. Antibodies used were anti-human IL-1 beta Ab (recognizes p17 and p31; Santa Cruz Biotechnology, Santa Cruz, CA) and antihuman beta-actin Ab (BD Biosciences).

**Real-time Reverse-transcription Polymerase Chain Reaction**

Total RNA of cells was isolated with an easy-BLUE total extraction kit (iNIRON Biotechnology, Sungnam, Korea), and complementary DNA was synthesized from 1 μg extracted RNA with M-MLV reverse transcriptase (Promega, Madison, WI) and dNTP mixture (Takara; Otsu, Shiga, Japan). Complementary DNA (2 μL) was mixed with 10 μL SYBR Applied Biosystems, Warrington, UK) and primer pairs (10 pmol each) in a 20-μL reaction volume. After incubation at 95°C for 10 minutes, the mixture was subjected to polymerase chain reaction for 40 cycles comprising a denaturation step at 95°C for 15 seconds and an annealing and extension step at 60°C for 1 minute. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping reference gene to normalize expression levels. The sequences of primers used were as follows: 5’-GCC AAT CTT CAT TGC TCA AGT GTC-3’ and 5’-TTG CTG TAG TGG TGG GA-3’ for IL-1 beta and 5’-GTC GCC AGC GCA GGC-3’ and 5’-TGA AGG GGT CAT TGA CA-3’ for GAPDH.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

The culture supernatants were subjected to determine IL-1β levels using ELISA assay kits (R&D Systems) according to the manufacturer’s protocol.

**Cell Death Assay**

Caspase-1 can generate pores in the plasma membrane, leading to cytoplasmic lactate dehydrogenase (LDH) release and propidium iodide (PI) uptake, both of which are characteristics of pyroptosis. Cell death was measured with an LDH cytotoxicity assay kit (BioVision, Palo Alto, CA). Cytotoxicity was calculated as the LDH release normalized to the maximum LDH release obtained when cells were treated with the cell lysis solution included in the LDH cytotoxicity assay kit. To quantify PI-positive cells, cells were double stained with PI (30 μmol/L, Sigma-Aldrich) and Hoechst33342 stain (10 μmol/L; Molecular Probes, Eugene, OR) for 30 minutes. Cells were fixed with 4% formaldehyde for 15 minutes and washed twice with PBS and chilled distilled water. Cells were mounted with aqueous/dry mounting medium (Biomed, Foster City, CA). Images of PI-positive cells were acquired by confocal laser scanning microscopy (LSM 700; Carl Zeiss, Jena, Germany), and the number of cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD).

**Measurement of Adenosine Triphosphate Release Induced by E. faecalis**

THP-1 cells were treated with E. faecalis (MOI of 1, 5, 20, and 50) or 0.5 μg/mL Pam3CSK₄ for 1.5 hours. The extracellular adenosine triphosphate (ATP) level was determined using an ATP bioluminescence assay kit according to the manufacturer’s protocol (Roche, Mannheim, Germany). Culture supernatants were mixed with 0.3% trichloroacetic acid and were kept at 4°C for 30 minutes. After adding 4 volumes of 250 mMol/L Tris-acetate (pH = 7.75), luciferase reagent was added to the mixture, and luminescence was measured using a GloMax 96 Microplate Luminometer (Promega).

**Results**

**E. faecalis Activates Caspase-1 in Macrophages**

Caspase-1 activation and IL-1 beta secretion by THP-1 macrophages were measured by immunoblotting of culture supernatants, whereas pro–IL-1 beta expression was measured by immunoblotting of cell lysates. Activation of caspase-1 protein was determined by the appearance of cleaved caspase-1 product (p20 subunit) in culture supernatants. As shown in Figure 1A, E. faecalis induced caspase-1 activation and IL-1 beta secretion in a dose-dependent manner. Increased IL-1 beta secretion was also detected by
enzyme-linked immunosorbent assay (Fig. 1B). Because IL-1 beta secretion in macrophages is preceded by pro–IL-1 beta expression, we expected that *E. faecalis* would induce pro–IL-1 beta production. As shown in Figure 1A, dose-dependent pro–IL-1 beta expression was detected in lysates of THP-1 cells stimulated with *E. faecalis*. We also confirmed pro–IL-1 beta expression at the RNA level by real-time reverse-transcription polymerase chain reaction (Fig. 1C). To confirm that *E. faecalis*–induced IL-1 beta secretion was caused by caspase-1 activation, cells were pretreated with the caspase-1 inhibitors Z-YVAD-FMK and Ac-YVAD-CHO before *E. faecalis* treatment. As shown in Figure 2, these inhibitors significantly inhibited *E. faecalis*–induced IL-1 beta secretion. These results indicate that *E. faecalis* induces IL-1 beta secretion in macrophages by the simultaneous induction of pro–IL-1 beta expression and processing of pro–IL-1 beta by caspase-1 activation. Pam3CSK4 was used as a positive control for pro–IL-1 beta expression via the Toll-like receptor 2 and caspase-1 activation via the NLRP7 inflammasome (26).

**Figure 1.** *E. faecalis* induced caspase-1 activation and IL-1 beta secretion. THP-1 macrophages were infected with live *E. faecalis* (MOI of 1, 5, and 20) or Pam3CSK4 (0.1 μg/mL and 0.5 μg/mL) for 6 hours. (A) Caspase-1 and IL-1 beta secreted into the culture supernatants (sup) and procaspase-1, pro–IL-1 beta, and beta-actin in the cell lysates (cell) were detected by immunoblotting. (B) Secreted IL-1 beta was measured by enzyme-linked immunosorbent assay and (C) messenger RNA expression of pro–IL-1 beta was measured by real-time reverse-transcription polymerase chain reaction. *P < .05 compared with the nontreated control.

**Figure 2.** *E. faecalis*–induced IL-1 beta secretion was inhibited by caspase-1 inhibitors. THP-1 cells were pretreated with caspase-1 inhibitors Z-YVAD-FMK and Ac-YVAD-CHO for 30 minutes before stimulation with *E. faecalis* for 6 hours. IL-1 beta secretion was detected by enzyme-linked immunosorbent assay. *P < .05 compared with the nontreated control and #P < .05 compared with *E. faecalis*– or Pam3CSK4-treated cells.
E. faecalis Induces Pyroptosis

Because pyroptosis is caspase-1–dependent inflammatory cell death, we tested whether *E. faecalis* induced caspase-1–dependent cell death by measuring LDH release and PI staining. As shown in Figure 3A, *E. faecalis* induced LDH release in a dose-dependent manner in THP-1 macrophages. In addition, *E. faecalis*–stimulated cells were stained with PI, whereas nonstimulated cells were not (Fig. 3B). Caspase-1 inhibitor Ac-YVAD-CHO inhibited *E. faecalis*–induced PI uptake. These results indicated that *E. faecalis* induces pyroptosis.

E. faecalis Induces ATP Release, Resulting in Caspase-1 Activation

Stressed or infected host cells can release ATP as a danger signal, and high concentrations of extracellular ATP are known to activate caspase-1 via the NLRP3 inflammasome by binding to the P2X7 receptor (27). *E. faecalis* induced ATP release in a dose-dependent manner (Fig. 4A), and an ATP receptor antagonist oxATP inhibited *E. faecalis*–induced caspase-1 activation and IL-1 beta secretion (Fig. 4B).

Discussion

In this study, we showed that *E. faecalis* can induce caspase-1 activation and pro–IL-1 beta expression, which are prerequisites for IL-1 beta secretion. IL-1 beta plays a critical role in host defense against bacterial infection. IL-1 beta is tightly controlled by separate regulation of pro–IL-1 beta expression and its processing to a mature form (28). Because THP-1 cells, which can differentiate into macrophages, have been widely used to study the separate regulation of IL-1 beta expression and secretion (28, 29), we used THP-1 macrophages to assess the ability of *E. faecalis* to induce both pro–IL-1 beta expression and caspase-1 activation. Simultaneous activation of these 2 steps can lead to overproduction of IL-1 beta and cause exaggerated inflammation, leading to tissue damage.

Increased IL-1 beta levels in inflamed pulp tissue have been detected by immunohistochemical staining and enzyme-linked immunosorbent assay, and increased IL-1 beta levels have been detected in cultured pulp fibroblasts treated with *Escherichia coli* lipopolysaccharide (30). *E. faecalis* (ATCC 19433) at a MOI of 300 significantly increased IL-1 beta messenger RNA expression in RAW264.7 cells, which are mouse macrophages (31). *E. faecalis* lipoteichoic acid increased IL-1 beta release in peripheral blood mononuclear cells.
Porphyromonas endodontalis lipopolysaccharide increased IL-1 beta messenger RNA expression and IL-1 beta release from human dental pulp cells without changing the activity of caspase-1 (33). Dental pulp is confined within a mineralized hard tissue (ie, dentin), and pulp cells are composed of fibroblasts, undifferentiated mesenchymal cells, odontoblasts, and other cell types including macrophages, lymphocytes, and dendritic cells (34, 35). All these cells participate in the inflammatory host defense response. Chronic inflammation persists in diseased pulp because of mechanical insults or bacterial invasion. Chronic pulp inflammation is characterized by the presence of increased numbers of macrophages, lymphocytes, and plasma cells. Our results indicate that E. faecalis infection of dental pulp may result in an excessively high IL-1 beta concentration, which can aggravate pulpal inflammation and cause tissue damage.

Our finding that caspase-1 is activated by E. faecalis increases our understanding of pulp inflammation, which is essential to provide effective treatment strategies. The identification of bacterial components at the molecular level and elucidation of mechanisms involved in activation of the inflammasome will provide insights into the pathogenesis of infectious diseases caused by multidrug-resistant E. faecalis.

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The authors deny any conflicts of interest related to this study.

References

Figure 4. E. faecalis–induced ATP is associated with caspase-1 activation. (A) THP-1 macrophages were treated with E. faecalis for 1.5 hours, and ATP levels in the culture supernatants were determined using an ATP bioluminescence assay kit. *P < .05 compared with the nontreated control. (B) THP-1 macrophages were pretreated with oxATP for 30 minutes before treatment with E. faecalis for 6 hours. Caspase-1 and IL-1 beta secreted into the culture supernatants (sup) and procaspase-1, pro–IL-1 beta, and beta-actin in the cell lysates (cell) were detected by immunoblotting.


