Calcium Hydroxide Inactivates Lipoteichoic Acid from *Enterococcus faecalis* through Deacylation of the Lipid Moiety

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Abstract

Introduction: Lipoteichoic acid (LTA) is a major virulence factor of *Enterococcus faecalis* that is closely associated with refractory apical periodontitis. Recently, we have shown that calcium hydroxide, a commonly used intracanal medicament, abrogated the ability of LTA to stimulate the production of tumor necrosis factor α in a murine macrophage line, RAW 264.7. Because calcium hydroxide could potentially modify the glycolipid moiety of LTA, we examined if calcium hydroxide inactivates LTA through deacylation of the LTA. **Methods:** LTA was prepared from *E. faecalis* by organic solvent extraction followed by chromatography with the hydrophobic-interaction column and the ion-exchange column. RAW 264.7 cells were stimulated with intact LTA or calcium hydroxide–treated LTA for 24 hours, and the productions of nitric oxide (NO) and chemokines interferon-gamma–induced protein (IP-10) and macrophage inflammatory protein-1α (MIP-1α) were determined. The glycolipid structure of LTA was analyzed using matrix-assisted laser desorption ionization-time of flight mass spectrometry and thin layer chromatography (TLC). **Results:** The production of NO, IP-10, and MIP-1α was augmented in LTA-stimulated cells, whereas no such effect was observed upon stimulation with calcium hydroxide–pretreated LTA. Mass spectrometry showed that intact glycolipids of LTA yielded distinct mass peaks at 930 to 1,070 mass over charge (m/z) units, corresponding to dihexosyldiacylglycerol consisting of two acyl chains with chain lengths of C_{16} to C_{22} and with one or two unsaturated double bonds. However, those peaks were not observed in the mass spectra of the calcium hydroxide–treated LTA. Furthermore, free fatty acids released from the calcium hydroxide–treated LTA were detected using TLC. **Conclusion:** We suggest that calcium hydroxide attenuates the inflammatory activity of *E. faecalis* LTA through deacylation of the LTA. *(J Endod 2011;37:191–196)*

Key Words

Apical periodontitis, calcium hydroxide, *Enterococcus faecalis*, intracanal medicament, lipoteichoic acid, matrix–assisted laser desorption ionization time of flight

Enterococci are commensal microorganisms that can be frequently found in the mucosal tissues in the oral cavity, gastrointestinal tract, and genital tract in humans. Enterococci are of clinical importance because they are the third most common nosocomial pathogens (1) and the cause of refractory apical periodontitis (2). They are often resistant to disinfectants and antiseptics because they can persist under harsh conditions, such as high alkalinity, because of their efficient use of proton pumps (3). To date, 12 enterococci species have been identified, and approximately 90% of the *Enterococcus* clinical isolates are *Enterococcus faecalis* (4).

E. faecalis expresses various virulence factors including lipoteichoic acid (LTA), peptidoglycan, aggregation substance, surface adhesins, sex pheromones, lytic enzymes such as gelatinase and hyaluronidase, and cytolysin (5). Of these virulence factors, LTA is considered one of the most important etiologic factors that are closely involved in pathogenicity based on the following aspects: (i) LTA is responsible for inflammatory responses and tissue damages (6); (ii) LTA purified from *E. faecalis* is able to induce proinflammatory cytokines and nitric oxide (NO) (7); (iii) *E. faecalis* LTA contributes to the bacterial adherence and biofilm formation that are essential for bacterial resistance to disinfectants, antibiotics, and host antimicrobial molecules (8); and (iv) opsonic antibodies to *E. faecalis* are predominantly generated against epitopes on the LTA (9).

LTA is an amphiphilic molecule composed of a glycolipid anchor together with either polyglycerolphosphates or polyribitolphosphates (10). Most gram-positive bacteria including *E. faecalis* express polyglycerophosphate-type LTA, whereas

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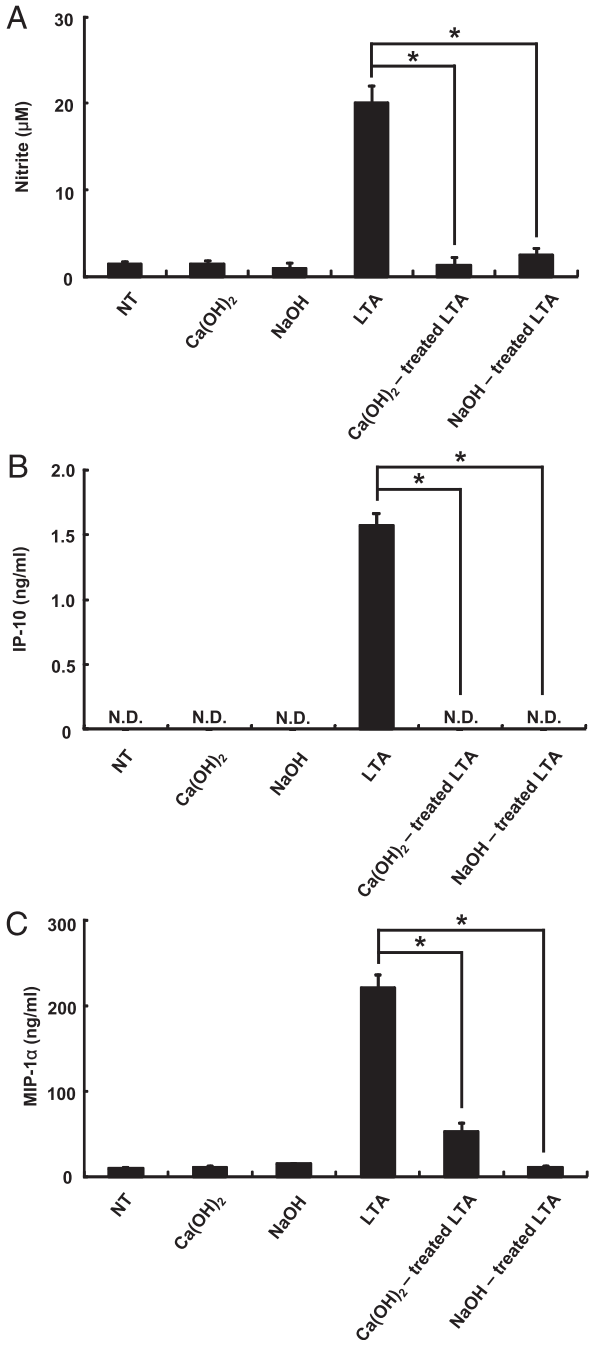

Figure 1. Calcium hydroxide abolishes the ability of *E. faecalis* LTA to stimulate the expression of inflammatory mediators. LTA from *E. faecalis* was treated with or without calcium hydroxide (25 mg/mL) at 37°C for 60 minutes. After pH neutralization, the calcium hydroxide–treated LTA was used for the stimulation of RAW 264.7 cells (1×10^6 cells/mL) for 24 hours. At the end of the incubation, culture media were collected and analyzed for the production of (*A*) NO, (*B*) IP-10, and (*C*) MIP-1α. Bars indicate mean values $\pm$ standard deviation, and asterisks are noted for data significantly ($p < 0.01$) different from the LTA-treated group. One of three similar results is shown. ND, not detected.

a few species such as *Streptococcus pneumoniae* produce polyribitolphosphate-type LTA (11). Studies on the structural and functional relationships suggest that glycolipid is mainly responsible for the immunostimulatory potential of LTA. In fact, delipidation of LTA by alkaline treatment almost completely abolishes the inflammatory and immunostimulatory capacities of LTA (12). In contrast, LTA with more acyl chains elicits stronger immunostimulating activities (13). Thus, the lipid moiety of LTA seems to be a molecular target for the control of infectious diseases.

Calcium hydroxide is widely used as an intracanal medicament to eliminate microorganisms in infected root canals because of its broad antimicrobial activity (14). Accumulating reports suggest that calcium hydroxide in aqueous solution releases hydroxide ion (OH^-), resulting in a high alkaline environment in which most bacteria cannot survive (15). Recently, we found that calcium hydroxide could attenuate the abilities of not only *E. faecalis* but also its LTA to stimulate murine macrophages. Indeed, LTA treated with calcium hydroxide could not stimulate the production of tumor necrosis factor α (TNF-α) or NO (16). However, the molecular mechanisms behind the inactivation of LTA caused by calcium hydroxide treatment have not been studied. The aim of the present study was to examine if calcium hydroxide deacylates LTA of *E. faecalis* resulting in the loss of immunostimulating activity with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and thin layer chromatography (TLC).

Materials and Methods

Reagents and Chemicals

Highly pure LTA was prepared from *E. faecalis* ATCC 29212 as previously described (7). Calcium hydroxide was purchased from DC Chemical Co. Ltd. (Seoul, Korea). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Invitrogen (Grand Island, NY) and HyClone (Logan, UT), respectively. Proteomics-grade water was purchased from Bio-RAD (Hercules, CA). Chloroform, acetic acid, and methanol were purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Treatment of *E. faecalis* LTA with Calcium Hydroxide

Previously, it has been shown that 25 mg/mL of calcium hydroxide or 0.2 N of sodium hydroxide completely abolished the ability of LTA to stimulate the expression of inflammatory mediators via macrophages (16, 17). Thus, LTA (450 or 900 μg) was incubated with calcium hydroxide (25 mg/mL), sodium hydroxide (0.2 N), or proteomics-grade water as a control for 60 minutes followed by neutralization to pH 7 with 1 N of HCl. Then, the samples were lyophilized and stored at −80°C until use.

Culture of RAW 264.7 Cells

The mouse macrophage cell line RAW 264.7 (TIB-71) was purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified incubator with 5% CO_2.

Measurement of Inflammatory Mediators

RAW 264.7 cells at 1×10^6 cells/mL were stimulated with intact or calcium hydroxide–treated LTA for 24 hours. At the end of the incubation period, culture media were collected and used for the determination of NO and chemokines, interferon-gamma–induced protein (IP-10), and macrophage inflammatory protein-1α (MIP-1α). For NO determination, the culture media were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine