Sodium Hypochlorite Inactivates Lipoteichoic Acid of Enterococcus faecalis by Deacylation

Sun Woong Hong, MS, * Jung Eun Baik, PbD, * Seok-Seong Kang, PbD, * Kee-Yeon Kum, DDS, PbD,  † Cheol-Heui Yun, PbD,  † and Seung Hyun Han, PbD*

Abstract

Introduction: Enterococcus faecalis is a pathogenic gram-positive bacterium frequently found in mucosal tissues of the oral cavity, gastrointestinal tract, and genitai tract (1). In addition to their role as a common nosocomial pathogen, the enterococci are major etiologic agents of persistent apical periodontitis (3, 4). Enterococcus faecalis is a prevalent species in periradicular lesions of teeth after endodontic treatment and is also involved in persistent apical periodontitis (3, 4). E. faecalis is commonly resistant to disinfectants and antibiotics because it is known to survive in severe environmental conditions such as high alkalinity (5).

Virulence factors such as lipoteichoic acid (LTA), lytic enzymes, cytolysin, adhesins, aggregation substance, and pheromones of E. faecalis are known to be involved in pathogenicity (6). Among these virulence factors, LTA is considered a major etiologic agent based on the induction of the inflammatory response and tissue damage (7, 8). For example, it has been shown that E. faecalis LTA (EfLTA) induces several inflammatory mediators such as nitric oxide (NO), macrophage inflammatory protein-1α (MIP-1α), and monocyte chemotactic protein-1 (7, 9). In addition, EfLTA is closely associated with bacterial adhesion and biofilm formation, which are responsible for resistance to disinfectants, antibiotics, and antimicrobial peptides (10).

LTA is an amphiphilic molecule consisting of a glycolipid anchor linked with polyglycerolphosphate or polylitolphosphate backbones (11). Most gram-positive bacteria including E. faecalis contain polyglycerolphosphate-type LTA, whereas a few gram-positive bacteria such as Streptococcus pneumoniae express polyrlitolphosphate-type LTA (12, 13). LTA exclusively activates Toll-like receptor 2 (TLR2), which leads to the production of various proinflammatory chemokines and cytokines (14). It has been well described that the glycolipid moiety of the LTA structure is critical for its immunostimulating potential. Delipidation of LTA completely abolishes the immunostimulating potential (15). On the other hand, LTA with more acyl chains shows higher immunostimulating activity (16). Therefore, the glycolipid moiety of the LTA structure can be a determinant to elicit immune responses including the production of inflammatory mediators.

Endodontic irrigants are used to eliminate causative bacteria including E. faecalis in the infected root canal. Sodium hypochlorite (NaOCl) has been widely used as an...
endodontic irrigant for effective bactericidal and nonspecific proteolytic activity (17) and is strongly alkaline and hypertonic (18). In addition, it is known to dissolve organic tissues containing fatty acids and lipids via a saponification reaction (19). Although the antibacterial effect of NaOCl is well recognized, it is poorly understood if NaOCl detoxifies a major virulence factor of gram-positive bacteria, LTA. Therefore, the aim of the study was to investigate whether NaOCl inactivates EfLTA, leading to the reduction of the inflammatory response.

**Materials and Methods**

**Bacteria, Reagents, and Chemicals**

*E. faecalis* ATCC 29212 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Brain-heart infusion medium was purchased from BD Biosciences (Franklin, NJ). NaOCl was purchased from Duksan Pure Chemicals (Ansan, Korea). All other reagents were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Fluorescein isothiocyanate–conjugated mouse monoclonal anti-human TLR2 antibody was purchased from Biolegend (San Diego, CA).

**EfLTA Preparation and Treatment with NaOCl**

*E. faecalis* was cultured in brain-heart infusion medium at 37°C, and the highly pure and structurally intact EfLTA was prepared as described previously (7, 20, 21). Unwanted biological molecules including endotoxin, nucleic acids, and proteins in the preparation were not detectable (data not shown). NaOCl-treated EfLTA was subjected to a PD-10 desalting column (GE Healthcare Life Sciences, Buckinghamshire, UK) to remove NaOCl and debris that may have formed by structural damage. After lyophilization, the concentration of EfLTA was determined using phosphate assay as previously described (22).

**Culture of RAW 264.7 Cells**

The murine macrophage cell line RAW 264.7 obtained from the ATCC was maintained in Dulbecco’s Modified Eagle’s Medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum ( Gibco, Waltham, MA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator with 5% CO2 (23).

**Measurement of NO and Proinflammatory Cytokines**

RAW 264.7 cells (1 × 10⁶ cells/mL) were treated with EfLTA or NaOCl- or sodium hydroxide (NaOH)-treated EfLTA for 24 hours. Then, the culture supernatants were collected, and the production of NO was measured as described previously (24, 25). The secretion of interferon-γ-inducible protein-10 (IP-10) and MIP-1α was determined using commercial enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instruction.

**Determination of TLR2 Activation**

CHO/CD14/TLR2 cells are Chinese hamster ovary cells constitutively expressing human CD14 and TLR2 that are stably transfected with a nuclear factor kappa B–dependent reporter plasmid to express membrane-bound CD35 in proportion to TLR2 activation (26). For the determination of TLR2 activation, the cells (3 × 10⁶ cells/mL) were stimulated with EfLTA (0, 1, 3, 10, or 30 µg/mL); NaOCl-treated EfLTA (0, 1, 3, 10, or 30 µg/mL); or Pam3CSK₄, a synthetic lipopeptide mimicking gram-positive bacterial lipoproteins that is widely used as a TLR2 ligand (27) (0.1 µg/mL), for 24 hours. To determine TLR2 activation, the cells were stained with FITC-conjugated mouse monoclonal antihuman TLR2, and then the expression was analyzed by flow cytometry using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

**Thin-layer Chromatographic Separation and Silver Staining**

Thin-layer chromatographic (TLC) separation was performed on a silica gel TLC plate (Silica gel 60; Merck, Whitehouse Station, NJ) (28), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining was performed as described previously (29).

**Statistical Analysis**

All experiments were conducted in triplicate, and treatment groups with EfLTA or NaOCl-treated EfLTA were compared with the nontreatment group; statistical significance was determined using the Student t test. An asterisk indicates a significant difference (P < .05) when compared with the nontreatment group.

**Results**

**When Compared with EfLTA, NaOCl-treated EfLTA Showed No or Little Immunostimulating Activities**

We previously showed that EfLTA induced the production of inflammatory mediators such as NO, IP-10, and MIP-1α in murine macrophages (9). To investigate whether the NaOCl treatment abolishes the immunostimulating activity of EfLTA, RAW 264.7 cells were treated with EfLTA or NaOCl-treated EfLTA for 24 hours. EfLTA induced a significant amount of NO, IP-10, and MIP-1α (Fig. 1A–C, respectively). However, NaOCl-treated EfLTA failed to induce the respective mediators, suggesting that NaOCl treatment completely abolished EfLTA-induced inflammatory responses. In addition, as a positive control, NaOH-treated EfLTA showed significantly less induction of NO, IP-10, and MIP-1α compared with EfLTA.

**NaOCl Reduced Ability of EfLTA to Induce NO Production at Low Concentrations and at Early Time Points**

To identify the optimal concentration and duration of NaOCl needed for inactivation, EfLTA was pretreated with 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, or 10⁻⁵ % NaOCl for 1 hour. Then, RAW 264.7 cells were incubated with NaOCl-treated EfLTA for 24 hours. As shown in Figure 2A, the production of NO was not induced by EfLTA treated with 10⁻³ to 10⁻⁴ % (ν/ν) NaOCl. However, it was noted that 10⁻¹ % NaOCl–treated EfLTA slightly increased the production of NO. This concentration of NaOCl appears to be insufficient to completely inactivate EfLTA and thus it might induce the expression of inducible nitric oxide synthase resulting in NO production (30). Furthermore, when EfLTA was treated with 5.25% NaOCl for various time points, a complete reduction of NO was observed as early as 0.5 minutes (Fig. 2B).

**Compared with EfLTA, NaOCl-treated EfLTA Fails to Activate TLR2**

We previously observed that EfLTA-induced inflammatory responses were through TLR2 activation (7). To examine whether the reduction of inflammatory responses was caused by impaired TLR2 activation, CHO/CD14/TLR2 cells were incubated with NaOCl-treated EfLTA to examine its activation. EfLTA increased TLR2 activation in a dose-dependent manner, as previously shown (7). However, NaOCl-treated EfLTA did not increase TLR2 activation (Fig. 3A and B), indicating that NaOCl treatment impaired TLR2 activation and that the reduction of inflammatory responses by NaOCl treatment is attributable to impaired TLR2 activation.
Structural Integrity of EfLTA Is Altered by NaOCl

To assess the structural integrity of EfLTA treated with NaOCl, we performed silver stain analysis. As shown in Figure 4, EfLTA showed a staining pattern displaying 2 distinct major bands between 7 and 20 kDa. However, NaOCl-treated EfLTA exhibited a smaller-sized band (<17 kDa), and the staining pattern was weak and fragmented. Furthermore, the <7-kDa band of NaOCl-treated EfLTA was similar to that of NaOH-treated EfLTA, suggesting that the structural integrity of EfLTA was altered upon treatment with NaOCl. To further investigate whether NaOCl deacylates the glycolipid moiety of the EfLTA structure, we performed TLC separation to examine the release of fatty acids from the EfLTA glycolipids treated with NaOCl. Figure 4B shows that various glycolipids from EfLTA were clearly detected in the TLC because of the distinct length and saturation degree of the fatty acid chains. In contrast, free fatty acids released from the NaOCl-treated EfLTA were detected, and the patterns were similar to those of calcium hydroxide— or NaOH-treated EfLTA, suggesting that EfLTA was deacylated by NaOCl treatment.

Discussion

We previously observed that EfLTA is responsible for the induction of inflammatory mediators by *E. faecalis* (7). Furthermore, calcium hydroxide, an endodontic medicament, reduced the immunostimulatory function of EfLTA (28). NaOCl has been widely used as an endodontic irrigant that exhibits potent antimicrobial action against pathogens in the root canal. In the present study, we further showed that treatment of EfLTA with NaOCl results in the impairment of immunostimulating activity by the delipidation of the EfLTA glycolipid moiety structure, potentially leading to a reduction of the inflammatory response in macrophages.

Accumulating results have pointed out that LTA from gram-positive bacteria including EfLTA directly binds to TLR2 (31) through its lipid moieties (32). Furthermore, LTA induced the production of...
inflammatory mediators such as tumor necrosis factor α and NO in macrophages (7, 20), whereas deacylated LTA failed to activate TLR2 for the production of tumor necrosis factor α and NO (20). Thus, the lipid moiety seems to be crucial for the induction of the inflammatory response. Our data also show that NaOCl-treated EfLTA impaired TLR2 activation and the induction of inflammatory mediators. In addition, TLC assays showed that NaOCl damages the EfLTA structure, potentially through deacylation. Therefore, NaOCl, which is one of the common endodontic irrigants, could effectively detoxify EfLTA by removing the lipid moieties.

The use of NaOCl for root canal treatment is mainly because of its low cost and ability to remove residual microorganisms (33). However, effective NaOCl solution concentrations (2.6%–5.25%) are cytotoxic (34); 1% NaOCl results in reduced mechanical strength of dentine, and 5% NaOCl may reduce flexural strength and the modulus of elasticity (35, 36). In this study, we found that ≤0.0001% NaOCl could effectively inhibit the EfLTA-induced inflammatory response. Notably, E. faecalis is known as a major etiologic pathogen causing persistent apical periodontitis (2), and EfLTA could contribute to the inflammatory response (7). Thus, based on the current results, the minimal concentration of NaOCl, which is far lower than the bactericidal concentration, could immediately inactivate EfLTA, probably to reduce the inflammatory response in the infected root canal.

It is believed that the complete removal of bacteria or bacterial toxins during endodontic treatment is beneficial for the prevention of persistent apical periodontitis. Previously, we showed that calcium hydroxide affects the immunostimulatory potential of EfLTA (28). Similarly, the current study shows that the complete inactivation of EfLTA...
is observed by NaOCl treatment at a minimal concentration within less than 1 minute. NaOCl is a common endodontic irrigant to eradicate microorganisms in the root canal and could be applied to rapidly detoxify EfLTA, a major virulence factor of Enterococcus faecalis.

Acknowledgments

Supported by grants from the National Research Foundation of Korea, which is funded by the Korean government (NRF-2015R1A2A1A15055453 and NRF-2015M2A2A6A01044894) and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, which is funded by the Ministry of Health and Welfare (HI14C0469), Republic of Korea.

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

References


