Probiotic Lactobacillus casei strain Shirota prevents indomethacin-induced small intestinal injury: involvement of lactic acid

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Submitted 18 September 2008; accepted in final form 14 May 2009

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used worldwide by millions of individuals for the treatment of musculoskeletal pain. Since serious upper gastrointestinal events such as gastroduodenal ulceration, perforation, and bleeding often limit the use of these drugs, strategies for prevention of such complications have been developed. Co-administration of gastroprotective drugs such as proton pump inhibitors (PPIs) and use of selective cyclooxygenase-2 (COX-2) inhibitors can reduce these gastrointestinal toxicities of NSAIDs (14, 28, 33). However, recent clinical studies using video capsule endoscopy have demonstrated that NSAIDs frequently injure the small bowel as well as the upper gastrointestinal tract and that PPIs have no inhibitory effect on NSAID-induced enteropathy (8, 16, 32). Although selective COX-2 inhibitors cause less small bowel injury than traditional NSAIDs (8, 9), it has been reported that the beneficial effects of selective COX-2 inhibitors on the small bowel may be lost with long-term use of them (15). Novel means of treatment of NSAID-induced enteropathy are thus urgently needed.

Toll-like receptor 4 (TLR4), a receptor of lipopolysaccharide (LPS) of gram-negative bacteria (22), plays a key role in the pathogenesis of a variety of tissue injuries via stimulation of multiple signaling events including the nuclear factor-κB (NF-κB) and mitogen-activated protein kinases pathways (12). LPS recognition and signaling by TLR4 requires the participation of an accessory molecule, MD2 (19), which is essential for translocation of functional TLR4 from the cytoplasm to the cell surface (20). We recently found that NSAIDs trigger inflammatory responses and injure the small intestine through activation of the LPS/TLR4 signaling pathway (31). Agents that possess antimicrobial activity or inhibit the TLR4 pathway might thus be useful for the treatment of NSAID-induced enteropathy.

It has recently been reported that use of probiotic bacteria may have therapeutic effects in gastrointestinal disorders such as inflammatory bowel disease (5, 23) and irritable bowel syndrome (21). The Lactobacillus casei strain Shirota (LcS) is a lactic acid bacterium originally isolated from humans and is a probiotic (18). LcS exerted antimicrobial activity against Escherichia coli in a murine model of urinary tract infection (2). Furthermore, LcS improved dextran sodium sulfate-induced colitis by inhibiting expression of proinflammatory cytokines in lamina propria mononuclear cells (17). In this study, we investigated whether LcS has probiotic effects on indomethacin-induced small intestinal injury in rats. We also examined the effects of LcS and related products such as L-lactic acid on small intestinal inflammation and LPS/TLR4-triggered inflammatory responses.

MATERIALS AND METHODS

Animals and induction of small intestinal injury. Specific-pathogen-free male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing ~200 g were used. They were housed in polycarbonate cages with paper chip bedding in an air-conditioned biohazard room with a 12-h light-dark cycle and had free access to food and water. All experi-
For assay of concentration of lactic acid, intestinal contents were homogenized in 1 ml of distilled water. The homogenate was placed in an Eppendorf tube and centrifuged at 10,000 rpm at 4°C for 10 min. A mixture of 0.9 ml of the resulting supernatant and 0.1 ml of 1.5 M perchloric acid was mixed well in a glass tube and allowed to stand at 4°C for 12 h. The suspension was then passed through a filter with a pore size of 0.45 μm (Kurabo Industries, Osaka, Japan). The HPLC was performed with a Waters system (Waters 432 Conductivity Detector; Waters, Milford, MA) equipped with two columns (Shodex Rspack KC-811; Showa Denko, Tokyo). The concentrations of lactic acid were calculated with a lactate standard curve and expressed as micromolar per gram small intestinal content.

Preparation of viable LcS, heat-killed LcS, and LcS culture supernatants. For preparation of viable bacteria, freeze-dried powders containing 10^9 CFU of LcS/g were suspended in sterile distilled water, whereas for preparation of heat-killed bacteria the harvested cells of LcS suspended in distilled water at a concentration of 10^9 CFU/ml were heated at 100°C for 30 min and then cooled on ice (2). LcS were grown aerobically in 10 ml of Bacto peptone (Becton, Dickinson, Tokyo, Japan) with glucose at a concentration of 10 mg/ml for 48 h at 37°C. Cell-free culture supernatants were obtained by centrifugation at 10,000 rpm for 10 min at 4°C. Centrifuged culture supernatants were passed through a sterile filter with a pore size of 0.45 μm (Kurabo Industries). Then the concentration of lactic acid in culture supernatants was determined by HPLC as described above, and the supernatants were adjusted to 10 mM lactic acid with culture medium just before use in in vitro experiments.

In vitro experiments. The human monocytic cell line THP-1 (Riken Cell Bank, Tsukuba, Japan) was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. THP-1 cells were plated overnight at 5 × 10^5/well in 24-well plates and then treated with 10 ng LPS 055:B5 (Sigma-Aldrich) in the presence or absence of L-lactic acid (0–15 mM), hydrochloric acid (4.2 mM, pH 6.8), or LcS culture supernatant containing 10 mM lactic acid for 1 or 2 h. Culture supernatants of THP-1 cells were collected for TNF-α assay, and THP-1 cells were collected for real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, or assay for quantification of NF-κB activation.

Determination of mRNA levels by RT-PCR. Total RNA was extracted from small intestinal tissue or THP-1 cells stimulated with 10 ng LPS for 2 h in the presence or absence of L-lactic acid or culture supernatant of LcS by use of an Isogen kit (Nippon Gene, Tokyo, Japan). Real-time quantitative RT-PCR analyses were performed as previously described (30). Expression of mRNAs for inflammatory genes was analyzed by real-time quantitative RT-PCR as described above. 

**Table 1. PCR primers and TagMan probes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and Probe</th>
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<tbody>
<tr>
<td>TFN-α</td>
<td>5'-CACAGAGAAAGTCAGCTGCT-3′</td>
</tr>
<tr>
<td></td>
<td>5'-TCTCAGCAAGCTGACAGT-3′</td>
</tr>
<tr>
<td>CINC-2α</td>
<td>5'-AACACGCTGCTGACAGT-3′</td>
</tr>
<tr>
<td></td>
<td>5'-CCGGCTCAGCTGACAGT-3′</td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-AGCGGCTCAGCTGACAGT-3′</td>
</tr>
<tr>
<td></td>
<td>5'-AGCGGCTCAGCTGACAGT-3′</td>
</tr>
<tr>
<td>TLR4</td>
<td>5'-ATCCAGCTGCTGACAGT-3′</td>
</tr>
<tr>
<td></td>
<td>5'-ACGGCTCAGCTGACAGT-3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-TCTCAGCAAGTCAGCTGCT-3′</td>
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<td></td>
<td>5'-TCTCAGCAAGTCAGCTGCT-3′</td>
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CINC-2α, cytokine-induced neutrophil chemoattractant-2α; iNOS, inducible nitric oxide synthase; TLR4, Toll-like receptor 4.
mediators including TNF-α, cytokine-induced neutrophil chemoattractant-2α (CINC-2α) (rat interleukin-8 homolog), inducible nitric oxide synthase (iNOS), and TLR4 was normalized for expression with GAPDH. The sequences of PCR primers and TaqMan probes are shown in Table 1.

Measurement of TNF-α production by THP-1 cells. THP-1 cells were stimulated with 10 ng LPS for 2 h as described above. Cell supernatants were collected and assayed for TNF-α production by standard sandwich ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Western blot analysis. THP-1 cells stimulated with 10 ng LPS in the presence or absence of 10 mM 1-lactic acid or LcS culture supernatant containing 10 mM lactic acid for 1 h were lysed on ice in a buffer containing 0.5% Nonidet P-40, 40 mM Tris·HCl pH 8.0, 120 mM NaCl, 1 mM PMSF, and 10 μg leupeptin/ml. Protein in the lysate was measured with a modified biocinchoninic acid method (BCA protein assay reagent kit, Pierce, Rockford, IL). Proteins were denatured with SDS sample buffer, subject to 10% SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline (TBS) containing 5% BSA and incubated overnight at 4°C with one of the following antibodies: anti-TLR4 (Cell Signaling Technology, Danvers, MA), anti-MD2 (Stressgen, Victoria, BC, Canada), total inhibitory TLR4 (Takara, Shiga, Japan), and expression of mRNAs for inflammatory mediators in rat small intestine 24 h after indomethacin administration. Indomethacin caused intestinal ulceration, and expression of mRNAs for inflammatory mediators was assayed in rats treated with viable *L. casei* strain Shirota for a week (B).

![Fig. 2. Histological findings in the small intestine 24 h after indomethacin administration. Indomethacin caused intestinal ulceration with destruction and necrosis of the epithelium and infiltration by inflammatory cells in controls (A) by 24 h, whereas less severe injury with mild inflammation was observed in rats treated with viable *L. casei* strain Shirota (B).](image)
A single dose of 10^9 CFU of viable LcS tended to inhibit injury \((P = 0.21)\), though not to a significant extent. Histologically, indomethacin caused intestinal ulceration with destruction and necrosis of the epithelium and infiltration by inflammatory cells in controls (Fig. 2A) by 24 h, whereas less...
Fig. 4. Effects of l-lactic acid and culture supernatant of *L. casei* strain Shirota on phosphorylation of I-κB-α and ERK and activation of NF-κB induced by LPS, and expression of Toll-like receptor 4 (TLR4) or MD2 in THP-1 cells. THP-1 cells were treated with 10 ng LPS for 1 h in the presence of 10 mM l-lactic acid (LA) or culture supernatant of *L. casei* strain Shirota containing 10 mM lactic acid. The cells were subjected to Western blot analysis or assay for quantification of NF-κB activation. A: a representative Western blot for phospho-I-κB-α, total I-κB-α, phospho-ERK, total ERK, TLR4, MD2, and β-actin. B: levels of expression of phospho-I-κB-α, total I-κB-α TLR4, and MD2 were normalized to β-actin, whereas level of expression of phosphor-ERK was normalized to that of total ERK. Each column represents means ± SE. N = 3. *P < 0.05, **P < 0.01 vs. LPS-nonstimulated group (control group), ##P < 0.05, ###P < 0.01 vs vehicle-treated group. C: translocation of p65, the subunit of NF-κB, from cytoplasm to nucleus was assayed using the ELISA-based TransAM NF-κB p65 kit. Each column represents means ± SE. N = 3. **P < 0.01 vs. LPS-nonstimulated group (control group), ###P < 0.01 vs vehicle-treated group.
severe injury with mild to moderate inflammation was observed in rats treated with viable LcS for a week (Fig. 2B).

**Inhibition of indomethacin-induced intestinal inflammation by LcS.** Indomethacin increased MPO activity by 4.2-fold and mRNA level for iNOS by 3.1-fold and also increased expression of TNF-α and CINC-2α at both mRNA and protein levels by 24 h (Table 2). Treatment with viable LcS at a dose of 10⁹ CFU for a week inhibited these increases. Indomethacin also significantly increased the level of expression of TLR4 mRNA. Repeated administration of LcS had no inhibitory effect on TLR4 expression.

Analysis of bacteria flora and lactic acid concentration in small intestine. As shown in Table 3, numbers of Enterobacteriaceae and Enterococci, which are gram-negative and gram-positive bacteria, respectively, were increased by 24 h after indomethacin challenge. Treatment with viable LcS at a dose of 10⁹ CFU once or once daily for a week had no inhibitory effect on the increase in numbers of these bacteria, whereas it decreased the number of Bacilli by 24 h. LcS was detected in the small intestinal content with both single and repeated administration of LcS by 3 h after indomethacin challenge. In the single-administration group, LcS was not detected by 24 h, whereas a comparatively large number of LcS remained detectable by 24 h in the repeat-administration group.

We next measured concentrations of lactic acid in the small intestine. Indomethacin challenge increased the concentration of lactic acid by 24 h. Single administration of viable LcS at a dose of 10⁹ CFU had no effect on lactic acid concentration, whereas repeated administration of LcS significantly increased the concentration of lactic acid, with prevention of increase in pH of small intestinal content (Table 4).

**Inhibition of LPS/TLR4 signaling pathway by l-lactic acid and LcS culture supernatant.** Treatment with 10 ng LPS markedly increased both TNF-α production and TNF-α expression in THP-1 cells (Fig. 3, A and B). l-Lactic acid inhibited TNF-α production and TNF-α mRNA expression in dose-dependent fashion, whereas 4.2 mM HCl, the pH of which is similar to that of 15 mM l-lactic acid, had no inhibitory effect on LPS-induced TNF-α overexpression. Culture supernatant of LcS containing 10 mM lactic acid exerted a stronger inhibitory effect on TNF-α expression at both protein and mRNA levels than l-lactic acid alone.

Western blotting showed that LPS induced phosphorylation of I-κB-α and ERK as well as I-κB-α degradation in THP-1 cells, whereas these changes were inhibited by LcS culture supernatant (Fig. 4, A and B). l-Lactic acid also inhibited phosphorylation of I-κB-α but did not inhibit the phosphorylation of ERK. Neither LcS culture supernatant nor l-lactic acid affected the expression of TLR4 or MD2.

LPS treatment for 1 h markedly activated NF-κB in THP-1 cells. l-Lactic acid at a concentration of 10 mM and culture supernatant of LcS containing 10 mM lactic acid strongly inhibited this activation (Fig. 4C).

**In vivo effects of l-lactic acid.** Finally, we examined the antiluferogenic effects of l-lactic acid. Since by 3 h after indomethacin challenge the mean concentration of lactic acid in the small intestinal contents in rats given LcS at a dose of 10⁹ CFU for a week was 13.8 ± 2.4 μM/g (= mM/kg, Table 4), we used l-lactic acid at concentrations of 1–15 mM. l-Lactic acid supplied in drinking water inhibited indomethacin-induced small intestinal injury in dose-dependent fashion, whereas 2.5 mM HCl, the pH of which is similar to that of 15 mM l-lactic acid (pH 2.7), did not prevent this injury (Fig. 5). Neither amount of food intake, amount of drinking, nor body weight differed among the groups.

**DISCUSSION**

In this study, we demonstrated that LcS had a strong inhibitory effect on indomethacin-induced small intestinal injury. Since a single dose of viable LcS, which resulted in transitory colonization of LcS in the small intestine, as did heat-killed LcS, failed to inhibit injury, sustained colonization of LcS during development of the injury may be needed for exertion of a probiotic effect on NSAID-induced small intestinal injury.

Gram-negative bacteria play a key role in the pathogenesis of NSAID-induced enteropathy (31). Probiotic *Lactobacillus* strains including LcS have been reported to possess antimicrobial activity (2, 27) exerted via various mechanisms (26). In particular, by producing metabolites such as lactic acid and thus lowering pH, *Lactobacillus* strains inhibit the growth of bacterial pathogens and sometimes even kill them (10). However, we found that repeated administration of LcS, which elevated lactic acid concentration in the small intestine, failed to inhibit the increase in number of *Enterobacteriaceae* (a gram-negative bacteria) as well as *Enterococci* (gram-positive bacteria), although it prevented elevation of pH of intestinal content. Prevention of injury by LcS may thus be independent of antibacterial activity.

Studies of the effects of lactic acid on inflammation have yielded conflicting results. Doudevani et al. (6) demonstrated a decrease in LPS-induced TNF-α mRNA expression and NF-κB DNA binding activity in human blood-derived macrophages incubated with lactated dialysis solution. Kellum et al.
(13) also demonstrated that lactic acid inhibited mRNA expression of iNOS, IL-6, and IL-10 as well as NF-κB DNA binding activity in LPS-stimulated RAW 264.7 cells, a mouse macrophage cell line, in dose-dependent fashion. In contrast, Jensen et al. (11) reported an increase in TNF-α expression in macrophages cultured with lactic acid. In this study, we demonstrated that l-lactic acid, an isomeric form of lactic acid produced by LcS, prevented NF-κB activation, phosphorylation of I-κB-α, and TNF-α expression in LPS-stimulated THP-1 cells. This anti-inflammatory effect of l-lactic acid may not be due to its pH-lowering effect, since 4.2 mM hydrochloric acid, the pH of which is similar to 15 mM l-lactic acid (pH 6.8), failed to inhibit overexpression of TNF-α in LPS-stimulated THP-1 cells.

I-κB has been recognized as an inhibitor of NF-κB (3), a major transcription factor that regulates the expression of a large number of genes that code for inflammatory mediators such as cytokines (4). NF-κB molecules are present in specific heterodimeric complexes present in inactive form in the cytoplasm of resting cells, with each molecule bound to one of the I-κB proteins. Upon stimulation, I-κB is phosphorylated, ubiquitinated, and degraded by the proteasome, allowing NF-κB to translocate to the nucleus (7). l-Lactic acid may thus prevent NF-κB activation through inhibition of phosphorylation of I-κB-α, leading to suppression of LPS-induced TNF-α expression in THP-1 cells.

Recently, Tien et al. (29) demonstrated that Caco-2 cells coincubated with L. casei exhibited stabilization of I-κB even after subsequent stimulation by Shigella or TNF-α and that L. casei induced downregulation of genes involved in the ubiquitination-degradation processes that are responsible for the degradation of I-κB in Caco-2 cells. The effects of lactic acid and culture supernatant of LcS on NF-κB activation may thus be mediated by effects on the ubiquitin proteasome system.

Repeated administration of viable LcS elevated lactic acid concentrations in small intestinal content and inhibited indomethacin-induced small intestinal damage with decrease in inflammatory responses such as neutrophil infiltration and cytokine expression, whereas single administration of viable LcS did not affect lactic acid concentration and did not inhibit injury. We also found that l-lactic acid given in drinking water markedly inhibited injury independent of its pH-lowering effect. Furthermore, we previously reported that neutralizing antibodies to TNF-α prevented such injury in rats (31). Collectively, these findings suggest that the anti-inflammatory effects of l-lactic acid produced by LcS may be a key mechanism by which LcS exerts probiotic effects on NSAID-induced small intestinal injury. Interestingly, Schurr et al. (24, 25) also reported a protective effect of lactic acid on neuronal injuries such as those resulting from hypoxia upon reoxygenation, suggesting that lactic acid may have preventive effects on a variety of tissue injuries.

Indomethacin challenge increased the concentration of lactic acid in small intestinal content by 24 h, although the mechanism by which it did so is unclear. However, indomethacin did not increase lactic acid concentration by 3 h. Since treatment with viable LcS for a week significantly increased the concentration of lactic acid by 3 h as well as 24 h after indomethacin challenge, elevation of lactic acid concentration in the early phase of injury may play an important role in inhibition of inflammatory responses and injury induced by indomethacin.

Notably, LcS culture supernatant containing 10 mM lactic acid had stronger anti-inflammatory effects on THP-1 cells stimulated with LPS than 10 mM l-lactic acid alone: culture supernatant and l-lactic acid inhibited production of TNF-α protein by 85 and 54%, respectively, and inhibited the level of expression of TNF-α mRNA by 83 and 37%, respectively. Furthermore, culture supernatant inhibited LPS-induced phosphorylation of ERK as well as I-κB-α without affecting expression of TLR4 or MD2. These findings suggest that, in addition to lactic acid, LcS may secrete metabolites that act as negative regulators of the ERK activation pathway. The anti-inflammatory effects of LcS may thus be mediated via synergistic effects of l-lactic acid and secreted molecules other than lactic acid.

In conclusion, our findings suggest that LcS protects against indomethacin-induced small intestinal injury in rats and that its probiotic effects may be mediated through anti-inflammatory effects of bacterial metabolites including l-lactic acid via interference with the signaling pathways triggered by LPS. Our findings clearly provide a rational basis for the clinical use of the probiotic LcS for prevention of NSAID-induced enteropathy.

GRANTS
This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

DISCLOSURES
T. Asahara and K. Nomoto are employed by Yakult Honsha.

REFERENCES