Saccharomyces boulardii Inhibits ERK1/2 Mitogen-activated Protein Kinase Activation Both in Vitro and in Vivo and Protects against Clostridium difficile Toxin A-induced Enteritis*

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Xinhua Chen1‡2, Efi G. Kokkotou1, Nasima Mustafa1, K. Ramakrishnan Bhaskar1, Stavros Sougioulitzis1, Michael O’Brien1, Charalabos Pothoulakis2, and Ciarán P. Kelly1†

From the 1Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, the 2Department of Pathology, School of Medicine, Boston University, Boston, Massachusetts 02215, and the 3Division of Pediatric Gastroenterology & Nutrition, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02215

Saccharomyces boulardii (Sb), a probiotic yeast, protects against intestinal injury and inflammation caused by a wide variety of enteric pathogens, including Clostridium difficile. Given the broad range of protective effects of Sb in multiple gastrointestinal disorders, we hypothesize that Sb modulates host signaling pathways involved in intestinal inflammatory responses. In this study, we found that Sb culture supernatant (Sbs) inhibits interleukin-8 production induced by C. difficile toxin A or IL-1β in human colonocyte NCM460 cells in a dose-dependent fashion. Furthermore, Sbs inhibited IL-1β and toxin A induced Erk1/2 and JNK/SAPK but not p38 activation in NCM460 cells. To test whether this inhibition also occurs in vivo, we used a previously established mouse ileal loop model. On its own, Sbs had no significant effect on basal fluid secretion or intestinal histology. However, Erk1/2 activation was significantly inhibited by Sbs in toxin A exposed mouse ileal mucosa. In control loops, toxin A increased fluid secretion (2.2-fold), histological score (3.3-fold), and levels of the chemokine KC (4.5-fold). Sbs pretreatment completely normalized toxin A mediated fluid secretion (p < 0.01), and histopathologic changes (p < 0.01) and substantially inhibited toxin A-associated KC increases (p < 0.001). In summary, the probiotic yeast S. boulardii inhibits C. difficile toxin A-associated enteritis by blocking the activation of Erk1/2 MAP kinases. This study indicates a new mechanism whereby Sb protects against intestinal inflammation and supports the hypothesis that Sb modulates host inflammatory signaling pathways to exert its beneficial effects.

Clostridium difficile causes antibiotic-associated diarrhea and colitis in animals and humans (1) and is one of the most common nosocomial infections (2). Toxin A, a 308-kDa protein (3), is a major virulence factor of C. difficile (4). Previously we showed that in human mononcytic cells, ERK2 and p38 MAP kinases are activated by C. difficile toxin A and are required for both IL-8 gene expression and cell necrosis (5). Injection of toxin A into rodent intestine causes fluid secretion, increased mucosal permeability and mucosal damage, and release of inflammatory mediators (6–8).

The yeast Saccharomyces boulardii (Sb) has been used for decades as a probiotic agent to prevent or treat a wide variety of human gastrointestinal disorders, including antibiotic-associated diarrhea and recurrent C. difficile diarrhea and colitis (9, 10). Sb may also be useful in preventing clinical relapse in Crohn disease (11). However, the mechanisms whereby Sb exerts its beneficial effects have not been fully characterized. Several studies indicate that Sb may utilize multiple different mechanisms for its protective effects (12). These include inhibition of pathogen adhesion (13), strengthening of enterocyte tight junctions (14, 15), neutralization of bacterial virulence factors (16, 17), and enhancement of the mucosal immune response (18–20). As intestinal inflammation is a common characteristic of C. difficile colitis, enterocolitis caused by many other human enteric pathogens, and Crohn’s disease, the broad-ranging beneficial effects of S. boulardii led us to hypothesize that this probiotic yeast may act through modulation of host signaling pathways that regulate the intestinal mucosal inflammatory response. To study this, we tested the effect of Sb on MAP kinase signaling in human colonic epithelial cells in vitro as well as in mouse ileal loops in vivo.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human colonic epithelial cells NCM460 were maintained in M3D medium (INCELL Corp., San Antonio, TX), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 incubator at 37 °C. Cells were cultured in a 37 °C humidified incubator.
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with 5% CO₂. Toxin A was purified to homogeneity from C. difficile strain 10463 (ATCC, Manassas, VA) as described previously (21). Recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN). Antibodies against MAP kinases (ERK1/2, P38, JNK/SAPK), and against MAP-specific MAP kinase were purchased from Cell Signaling Technology (Beverly, MA).

**Preparation of S. boulardii Culture Supernatant (SbS)—**Lyophilized Sb was provided by Biocodex Laboratories, Montrouge, France and was cultured in RPMI 1640 cell culture medium (100 mg/ml) for 24 h in 37 °C. The suspension was then centrifuged at 9000 × g for 15 min and the supernatant collected. The supernatant was then passed through a 0.22-μm filter (Fisher Scientific) to remove yeast cells. We and others reported previously that Sb secretes large molecular weight proteins (>50 kDa) possessing biologic activity (16, 22–24). To exclude activity arising from those molecules, we passed the filtered supernatant through a 10-kDa cutoff filter (Millipore, Bedford, MA). The resulting fraction was termed Sb supernatant (SbS).

**IL-8 Enzyme-linked Immunosorbent Assay—**IL-8 protein levels were measured by an enzyme-linked immunosorbent assay, as described previously (25). In brief, after incubation, aliquots of conditioned media were added to NUNC Maxisorp 96-well plates (Nunc Inc., Naperville, IL) precoated with goat anti-IL-8 (R&D Systems) overnight at 4 °C and incubated for 30 min at room temperature. IL-8 release was then assayed using a biotinylated anti-IL-8 antibody (R&D Systems). The presence of SbS in the cell supernatant does not interfere with the cytokine assay directly as previous reported (26). Results were expressed as mean ± S.E. (ng/ml). At least three independent experiments were performed for each experimental condition, each with triplicate measurements.

**Western Blotting—**NCM460 cells were stimulated with Toxin A (3 nM) or recombinant human IL-1β (10 ng/ml), in the presence or absence of SbS for different time periods. Treated cells were then lysed in a lysis buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.01% bromphenol blue, and 1% 2-mercaptoethanol). Equal amounts of cell extract were fractionated by 10% SDS-PAGE, and proteins were transferred onto nitrocellulose membranes (Bio-Rad) at 300 mA for 3 h. Membranes were blocked in 5% nonfat dried milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) and then incubated with antibodies directed against ERK1/2, phospho-ERK1/2, JNK/SAPK, phospho-JNK/SAPK, P38, and phospho-P38 (Cell Signaling). Membranes were washed with TBST and incubated with horseradish peroxidase-labeled secondary antibodies for 1 h. The peroxidase signal was detected by Supersignal chemiluminescent substrate (Pierce), and the image of the signal was recorded by exposure to x-ray film (Fujiﬁlm, Tokyo, Japan).

**Toxin A Ileal Loop Experiments—**Mice were anesthetized with a mixture of ketamine (0.9 ml, 100 mg/ml) and xylazine (0.1 ml, 100 mg/ml) in 9 ml of saline at a dose of 0.15 ml/20 g of body weight. A laparotomy was then performed, and one 3- to 5-cm-long loop was formed at the terminal ileum as previously described (27). Mouse ileal loops were injected with 150 μl of SbS or vehicle (RPMI). After 30 min, loops were injected with either 0.15 ml of 50 mM Tris buffer, pH 7.4, containing 10 μg of purified toxin A or Tris buffer alone (control). The abdomen was then closed, and animals were placed on a heating pad at 37 °C for the duration of the experiment. At 0.5, 1, and 2 h, some animals (n = 3 per group) were killed by CO₂ inhalation, and loops were collected for Western blot analysis. 4 h after toxin A injection, the remaining animals (n = 7 per group) were killed, and fluid secretion was estimated as the loop weight-to-length ratio as described (27). Ileal tissue samples were fixed in forma-
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RESULTS

SbS Inhibited IL-8 Secretion by IL-1β or C. difficile Toxin A-stimulated Human Colonic Epithelial Cells—NCM460 cells, a human colonocyte cell line, were serum-starved overnight and then stimulated with recombinant human IL-1β (10 ng/ml) or toxin A (1 µg/ml) in the presence or absence of serial 2-fold dilutions of SbS. After 12-h incubation, the cell conditioned media were collected and IL-8 protein levels measured by ELISA. As illustrated in Fig. 1 resting NCM460 cells released little IL-8 protein while stimulation with IL-1β (Fig. 1A) or toxin A (Fig. 1B) markedly increased IL-8 protein production. The presence of SbS significantly reduced IL-8 production in toxin A or IL-1β-stimulated NCM460 cells, in a dose-dependent fashion.

SbS Inhibited IL-1β-induced Erk1/2 and JNK/SAPK but Not p38 Activation in NCM460 Cells—MAP kinases regulate cell survival and cytokine production in response to stress and are an important pathway for the regulation of IL-8 production. As shown in Fig. 2, IL-1β induced Erk1/2, JNK/SAPK, and p38 phosphorylation in NCM460 cells at various time points. The presence of SbS inhibited activation of Erk1/2 and JNK/SAPK but had no evident effect on p38 activation.

SbS Inhibited Toxin A-induced Erk1/2 and JNK/SAPK but Not p38 Activation in NCM460 Cells—Previously we reported that C. difficile toxin A induces MAP kinase phosphorylation in THP-1 monocytic cells. Activation of p38 MAP kinase by toxin A in enterocytes has also been reported. However, the ability of toxin A to activate Erk or JNK MAP kinases in human colonocytes has not been reported. As shown in Fig. 3, toxin A (3 nM) induced Erk1/2, JNK/SAPK, and p38 phosphorylation in NCM460 cells at various time points. The presence of SbS inhibited activation of Erk1/2 and JNK/SAPK but had no evident effect on p38 activation.

SbS Inhibited Activation of Erk1/2 by Toxin A in Mouse Ileal Loops—Mouse ileal loops with 30 min of pretreatment with SbS or RPMI (150 µl) were injected with toxin A (10 µg). After 0.5, 1, and 2 h, mouse loop tissues were collected. Homogenized tissues were run on SDS-
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PAGE and blotted for the presence of MAP kinases. As shown in Fig. 4, toxin A strongly induced Erk activation in vivo at 0.5 and 1 h after toxin inoculation. SbS pretreatment significantly inhibited that activation at both the 0.5 and 1 h time points (p < 0.01). After 2 h there was minimal persisting Erk activation by toxin A. Toxin A treatment also increased JNK/SAPK activation in ileal mucosal tissues after 0.5 h and this was again prevented by SbS pretreatment (Fig. 5). There was no significant increase in JNK/SAPK activation at the 1 and 2 h time points. SbS had no evident effect on p38 activation at any of the three time points examined (Fig. 5), consistent with what we observed in our in vitro experiments.

SbS Protected against Toxin A-mediated Fluid Secretion, Tissue Damage, and Increased KC Levels in Mouse Ileal Loops—In vehicle-pretreated loops, toxin A increased fluid secretion by 2.2-fold as quantified by the loop weight:length ratio, compared with control loops (Fig. 6). Pretreatment with SbS inhibited this enterotoxigenic effect of toxin A (buffer: 102.6 ± 12.5; buffer + SbS, 90.3 ± 8.3; toxin A, 224.1 ± 44.8; toxin A + SbS, 90.1 ± 15.1; p < 0.01, n = 7). SbS itself has no evident effect on base-line fluid secretion (Fig. 6).

The degree of histological damage in mouse ileal loops (epithelial necrosis, mucosal congestion and edema, and neutrophil infiltration) were quantified using a scoring system, as described previously (27). As illustrated in Fig. 7, toxin A caused a 3.3-fold increase in the histological injury score compared with control. Pretreatment with SbS protected against this histological injury (buffer: 1.8 ± 0.9; buffer + SbS, 1.8 ± 1.0; toxin A, 5.8 ± 1.0; toxin A + SbS, 1.5 ± 0.3; p < 0.01, n = 7).

Since we found that SbS inhibited toxin A-induced IL-8 chemokine secretion in human colonocytes, we measured levels of the chemokine KC, the mouse ortholog of IL-8, in mouse ileal loops. As shown in Fig. 8, toxin A increased KC concentrations in the ileal mucosa by 4.5-fold. Pretreatment of the ileal loops with SbS prevented toxin A-induced KC up-regulation. SbS alone did not alter KC concentrations (buffer, 304 ± 81; buffer + SbS, 143 ± 71; toxin A, 1390 ± 233; toxin A + SbS, 426 ± 162 pg/mg total protein, p < 0.001, n = 7).
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DISCUSSION

C. difficile is a Gram-positive bacterium responsible for a variety of gastrointestinal diseases including antibiotic-associated diarrhea and pseudomembranous colitis (4). S. boulardii has been used safely for many decades and in millions of patients to protect against intestinal injury, inflammation, and secretion caused by a wide variety of bacterial enteric pathogens including C. difficile (9, 10). In a recent controlled trial S. boulardii was also effective in reducing clinical relapse in Crohn disease (11).

The specific cellular and molecular mechanisms for the protective and therapeutic effects of probiotic agents such as S. boulardii in enteric infections and in inflammatory bowel disease in humans are not well known. The broad-ranging beneficial effects of S. boulardii led us to hypothesize that this probiotic yeast may act through modulation of host signaling pathways that regulate intestinal mucosal inflammatory, secretory, and barrier functions. We report here that S. boulardii exerts its anti-inflammatory activity, in part, by modulating host MAP kinase signaling pathways, both in vitro and in vivo.

We showed that S. boulardii conditioned media inhibited IL-8 production induced by either IL-1β or toxin A in a dose-dependent fashion (Fig. 1). Our recent report indicated that the inhibitory effect of SbS is reversible and not associated with loss of cell viability or protein production (26). It is known that MAP kinases regulate IL-8 production in response to proinflammatory cytokines or lipopolysaccharide (31, 32) as well as C. difficile toxin A (5). We now show that the activation of Erk1/2 and JNK/SAPK by both IL-1β and toxin A are inhibited by the presence of SbS in human colonocytes (Figs. 2 and 3). SbS also significantly inhibits Erk1/2 activation in toxin A-treated mouse ileal loops (Fig. 4). A similar inhibitory effect is evident for JNK/SAPK activation but is significant only at early time points after exposure to toxin A (Fig. 5). Interestingly, SbS did not affect the activation of p38 in NCM460 cells stimulated by IL-1β or toxin A (Figs. 2 and 3) nor did it affect p38 activation in vivo (Fig. 5). Samples from the in vivo mouse ileal loop experiments used for Western blot consisted of homogenized full thickness ileal tissues. Thus, the activated MAPK signals that we detected were the sum total of signals from all cell types not just intestinal epithelial cells. Nonetheless, the degree of SbS inhibition of activation on these three MAP kinases in vivo are consistent with its effect in NCM460 cells in vitro with greatest inhibition of Erk1/2 kinase activation and no significant effect on p38 activation. Since these three main MAP kinase families form three parallel cascades that can be activated simultaneously or independently (33–35), this suggests that the upstream cellular target of SbS is more directly involved with Erk1/2 and JNK/SAPK and less or not at all with p38.

Recently we reported that Sb conditioned media also inhibited tumor necrosis factor-α or lipopolysaccharide stimulated IL-8 production from THP-1 transformed human monocytic cells and from gastric epithelial cells (26). Moreover, a report by Dahan et al. (36) suggests that the addition of S. boulardii whole yeast to T84 human enterocytes attenuates enteropathogenic Escherichia coli-induced NF-κB and MAP kinases activation through undefined mechanisms. Another study suggests S. boulardii stimulates PPAR-γ expression and reduces response of human colon cells to proinflammatory cytokines (37). These reports support the hypothesis that S. boulardii is capable of modulating host signaling events, independent of specific stimuli such as IL-1β, C. difficile toxin A (in this study), enteropathogenic Escherichia coli (15), tumor necrosis factor-α, or lipopolysaccharide (26). This general effect on host signaling may at least partially explain why S. boulardii can have therapeutic effects on such a wide variety of gastrointestinal disorders.

The phenomenon whereby S. boulardii attenuates host signaling events is not unique to this eukaryotic probiotic agent. Probiotic bacteria have been reported to alter critical host signaling events that regulate intestinal inflammation. One example is VSL#3 (composed of Lactobacillus and Bifidobacteria), which inhibits the activation of the NF-κB pathway in cultured intestinal epithelial cells (30). It has also been reported that Lactobacillus rhamnosus GG inhibits activation of the p38 MAP kinase by tumor necrosis factor, interleukin-1α, or γ-interferon but has no effect on the NF-κB pathway (29). These studies together with our data support the notion that beneficial effects of probiotics in enteric diseases may be mediated through modulation of host proinflammatory responses, thereby inducing or maintaining intestinal hyporesponsiveness to pathogenic determinants within the lumen of the gastrointestinal tract (28). Further studies of the molecular basis for probiotic regulation of the intestinal epithelium are relevant to our understanding the mechanisms of therapeutic effect of probiotic agents as well as the pathogenesis of infectious enterocolitis and inflammatory bowel diseases.

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