

Extracellular Signal-Regulated Kinase-Dependent Nitric Oxide Production from Macrophage-Like Cells by Lactic Acid Bacteria

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Abstract – Lactic acid bacteria (LAB) are considered as probiotics with immunostimulatory property. In this study, we investigated the molecular mechanism of its immunostimulating potency on macrophages using combined preparation of LAB (cpLAB). cpLAB is able to strongly stimulate nitric oxide (NO) production as well as inducible NO synthase (iNOS) expression from macrophage-like RAW264.7 cells. The cpLAB-induced NO release seemed to be mediated by extracellular signal-regulated kinase (ERK) but not p38 and C-Jun N-terminal kinase (JNK), since U0126, an ERK inhibitor, clearly suppressed NO production. cpLAB significantly diminished the binding of toll like receptor (TLR)-2 antibody up to 25%, implying that cpLAB-mediated activation of macrophages may be required for the functional activation of TLR-2, but not TLR-4. Therefore, our data suggest that cpLAB may directly allow macrophages to immunostimulating potency via activation of TLR-2 and ERK.

Keywords: Lactic acid bacteria, Nitric oxide, Extracellular signal-regulated kinase

INTRODUCTION

Macrophages are representative immune cells with the host defense functions such as 1) the removal of tumor cells 2) killing of pathogenic microorganisms or cancer cells, and 3) the processing and presentation of tumor or bacterial antigens to lymphocytes (Cutolo, 1999; Gordon and Read, 2002). Tumor necrosis factor (TNF)- α and nitric oxide (NO) are the key molecules in these macrophage-mediated defensive roles (Yrlid *et al.*, 2000). For producing these molecules, a series of signaling activation cascades linked from receptors such as toll-like receptor (TLR)-2 to transcriptional factors such as nuclear factor (NF)- κ B, as well as intracellular signaling machinery such as tyrosine kinases, serine/threonine kinase, phosphatidy-

linositol 3-kinase (PI3K)/Akt, and mitogen-activated protein kinases (MAPKs) are required (Trinchieri and Sher, 2007).

Lactic acid bacteria (LAB), noninvasive and nonpathogenic probiotics of Gram-positive bacteria to produce lactic acid, are a representative edible immunostimulatory material that is used for both medicinal and food purposes (Sanz *et al.*, 2007; Wells and Mercenier, 2008). Aggressive systemic studies led us to understanding the value of LAB as a useful biomaterial. Indeed, immunopotentiating, anti-cancer, anti-diarrhea, anti-bacterial (against *Helicobacter pylori*), and anti-allergic effects have been demonstrated under LAB treatment (Ljungh and Wadstrom, 2006; de Moreno de LeBlanc, *et al.*, 2007; Schabussova and Wiedermann, 2008). Although a lot of applications have been developed, exact molecular mechanisms of LAB-mediated immunostimulating roles have not been fully elucidated so far. In this study, we aimed to prove the activating roles and action mechanisms of LAB on macrophage-mediated innate immune response. To do this, the inductive mechanism of NO release from macrophages

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under treatment of combined preparation of LAB (cpLAB) was investigated by focusing on ERK activity.

MATERIALS AND METHODS

Materials

Combined preparation of lactic acid bacteria (cpLAB), a mixture of *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Lactobacillus casei*, *Bifidobacterium breve* (each 1×10^{10} cells/g) was kindly donated from Kim Jeong Moon Aloe Co. (Product name: Interseven, Seoul, Korea). Peptidoglycan (PGN), zymosan, Poly (I:C), pam3csk4, and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). U0126, SB203580, and SP600125 were obtained from Calbiochem. (La Jolla, CA). Antibodies to TLR-2 (Schroder *et al.*, 2000), and TLR-4 (MTS510) (Kawasaki *et al.*, 2003) were from PharMingen (San Diego, CA) and Serotec (Kidlington, Oxford, UK), respectively. Fetal bovine serum (FBS) and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 cells were purchased from the American Tissue Culture Center (Rockville, MD). All other chemicals were of Sigma grade. Phospho- or total forms of antibodies to ERK, p38, JNK, and β -actin were purchased from Cell Signaling (Beverly, MA).

Cell culture

RAW264.7 cells were maintained in RPMI1640 supplemented with 100 U/ml of penicillin, 100 μ g/ml of streptomycin and 10% fetal bovine serum. Cells were grown at 37°C with 5% CO₂.

Determination of NO production

After the preincubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, cpLAB was added for 24 h, as reported previously (Lee *et al.*, 2009). The nitrite in the culture supernatants was measured by adding 100 μ l of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μ l samples.

Table I. The primer sequences of the investigated genes in a RT-PCR analysis

Gene		Primer sequences
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'
	R	5'-GGCTGTGAGAGCCTCGTGGCTTTGG-3'
GAPDH	F	5'-CACTCACGGCAAATTCACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

RT-PCR

For the evaluation of LPS-inducible gene mRNA expression levels, the total RNA from cpLAB-treated (or untreated)-RAW264.7 cells was prepared by adding TRIzol Reagent (Gibco BRL), according to the manufacturer's protocol. Semiquantitative RT reactions were conducted using MuLV reverse transcriptase as reported previously (Lee *et al.*, 2009). The primers (Bioneer, Seoul, Korea) used in this experiment are indicated in Table I (F: forward, R: reverse).

Immunoblotting

Cells (5×10^6 cells/ml) were lysed in lysis buffer (in mM: 20 TRIS-HCl, pH 7.4, 2 EDTA, 2 EGTA, 50 β -glycerophosphate, 1 sodium orthovanadate, 1 dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 10 μ g/ml pepstatin, 1 benzimidazole and 2 hydrogen peroxide) for 30 min under rotation in a cold-room. The lysates were clarified by centrifugation at 16,000 \times g for 10 min at 4°C. Soluble cell lysates were immunoblotted and phosphotyrosine, ERK or phospho-ERK were visualized as reported previously (Lee *et al.*, 2009).

Flow cytometric analysis

The surface level of TLR-2 (1:50 dilution) in RAW264.7 cells was determined by flow cytometric analysis, as reported previously (Cho *et al.*, 2001). Stained cells were analyzed on a FACScan.

Statistical analysis

A Student's *t*-test and a one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as means \pm standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. *p* values of 0.05 or less were considered to be statistically significant.

RESULTS AND DISCUSSION

Improving host immune defense system is importantly regarded for children, elderly people and cancer patients. Edible immunostimulating materials such as mushroom, mistletoe and LAB are familiar to us for this purpose. Due to numerous studies, so far, LAB has been known to have various immunostimulatory activities such as anti-cancer and anti-bacterial effects (Ljungh and Wadstrom, 2006; de Moreno de LeBlanc *et al.*, 2007; Schabussova and Wiedermann, 2008). However, the molecular mechanisms by which the LAB is able to up-regulate macrophage-mediated

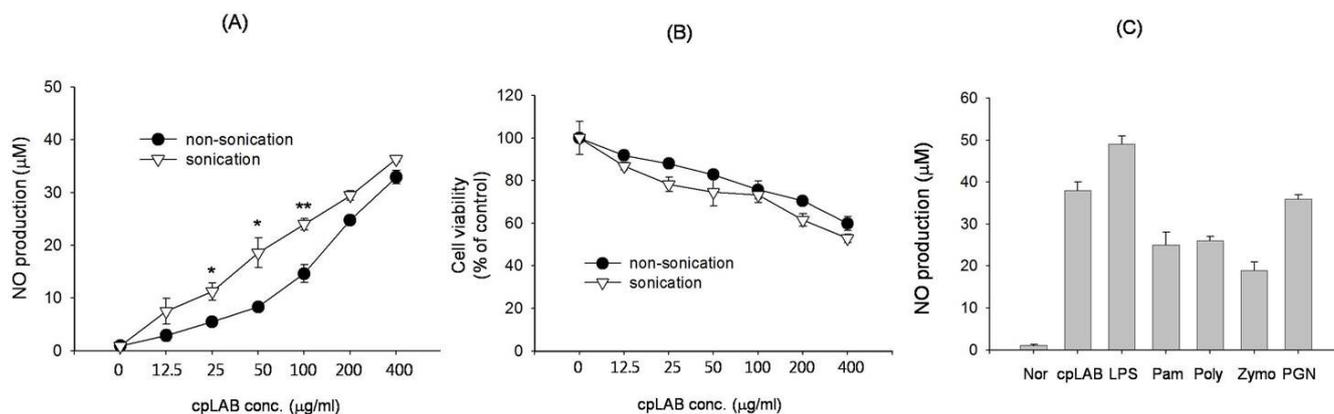


Fig. 1. Effect of cpLAB on NO production in RAW264.7 cells. (A) RAW264.7 cells (1×10^6 cells/ml) were stimulated by sonicated or non-sonicated cpLAB for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. (B) The viability of RAW264.7 cells (1×10^6 cells/ml) stimulated by sonicated or non-sonicated cpLAB for 24 h was measured by MTT assay. (C) RAW264.7 cells (1×10^6 cells/ml) were stimulated by non-sonicated cpLAB (400 µg/ml), LPS (2 µg/ml), Pam (pam3CSK4: 10 µg/ml), Poly [Poly (I:C): 10 µg/ml], Zymo (zymosan: 100 µg/ml), or PGN (peptidoglycan: 10 µg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent. Data represent mean \pm SEM of three independent observations performed in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared to normal.

host immunity remain largely unelucidated. In this study, therefore, functional activation mechanism of macrophages by LAB was carefully explored.

It has been reported that LAB is able to up-regulate or down-regulate NO production from macrophages (Hur *et al.*, 2004; Kim *et al.*, 2007; Lee *et al.*, 2008). To test whether cpLAB is able to modulate NO production, therefore, we first examined its NO modulatory ability using murine macrophage-like RAW264.7 cells. Under our conditions, cpLAB was revealed to have strong NO production effect (Fig. 1), as seen in the cases of enzymatically digested lactobacillus strains or lactic acid bacteria from Kimchi (Hur *et al.*, 2004; Kim *et al.*, 2007). Thus, cpLAB treated to RAW264.7 cells dose-dependently increased NO production (Fig. 1A) and consequently suppressed cell viability (Fig. 1B), mainly altered by produced NO (Heneka *et al.*, 1998). To compare the difference of NO producing effect by cpLAB between intact bacterium or lysed conditions, sonicated or non-sonicated forms of cpLAB were treated to RAW264.7 cells for allowing NO production. Other groups and our data suggested that the immunogenic activity of LAB derived proteins seems not to be altered by sonication, acid treatment and enzymatic digestion, although NO production was significantly distinct at several concentrations. Thus, enzymatically digested bacteria also enhanced NO production (Kim *et al.*, 2007), and cytoplasmic or cell wall products strongly induced NO production (Tejada-Simon and Pestka, 1999). Nonetheless, the fact that sonicated form of cpLAB more strongly enhanced NO production

LPS (2.5 µg/ml)	-	+	-
cpLAB (0.1 mg/ml)	-	-	+

iNOS



β-actin

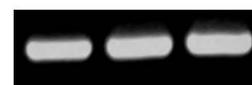


Fig. 2. The effect of cpLAB on the expression of iNOS in RAW264.7 cells. RAW264.7 cells (1×10^7 cells/ml) were incubated with cpLAB (100 µg/ml) or LPS (2.5 µg/ml) for 6 h. The mRNA level of iNOS was determined by semi-quantitative RT-PCR. The results show one experiment out of three.

than intact form, at the ranges of 10 to 200 µg/ml (Fig. 1A) implies that other cellular components not released in the case of intact cpLAB may also participate in activation process of macrophages. The NO production effect of cpLAB seems to be considerable in terms of its immunostimulating potency, compared to those of other immunostimulating agents. Thus, macrophages were able to secrete NO under treatment of LPS, poly (I:C), pam3csk4, mushroom-derived polysaccharide (β-glucan), zymosan, and peptidoglycan (PGN) in addition to cpLAB. Of them, LPS displayed the most strong NO producing effect. cpLAB also highly released NO from RAW264.7 cells, as much as other stimuli did (Fig. 1C). The production of NO

was linked to transcriptional activation of iNOS. Namely, iNOS level was remarkably appeared at 6 h incubation of cpLAB, as in the case of LPS (Fig. 2), indicating that upstream signaling pathways may be required for iNOS expression.

Although numerous signaling cascades are reported in inflammatory responses, the involvement of MAPK was explored in this study, since there were no relevant reports

in cpLAB-induced NO production conditions. Of MAPK inhibitors, U0126 strongly suppressed cpLAB-induced NO production (Fig. 3A), suggesting that ERK may play a critical role in managing NO production. Indeed, phospho-ERK was nicely observed by sonicated or non-sonicated cpLAB treatment at 30 to 60 min, while LPS triggered the phosphorylation of ERK at 15 min, according to immunoblotting analysis (Figs. 3B and 3C). In contrast, the

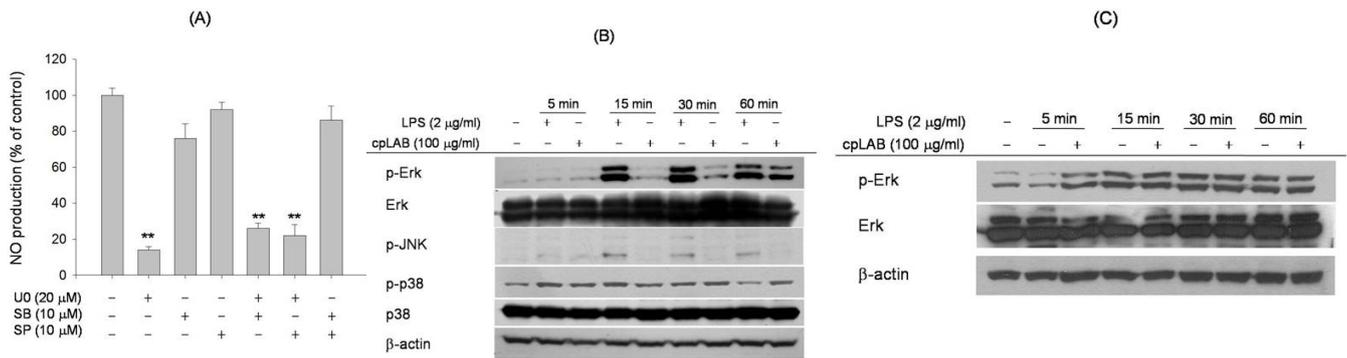


Fig. 3. The involvement of MAPK in cpLAB-induced NO production in RAW264.7 cells. (A) RAW264.7 cells (1×10^6 cells/ml) were pre-treated by MAPK inhibitors [U0126 (20 μM), SB203580 (10 μM) and SP600125 (10 μM)] in the presence of cpLAB (100 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. (B and C) RAW264.7 cells (5×10^6 cells/ml) were stimulated by non-sonicated (B) or sonicated (C) cpLAB (100 μg/ml) or LPS (2 μg/ml) for indicated times. After immunoblotting, the levels of phospho- or total ERK, p38, JNK and β-actin were identified by their antibodies. The results show one experiment out of three. Data represent mean ± SEM of three independent observations performed in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared to control.

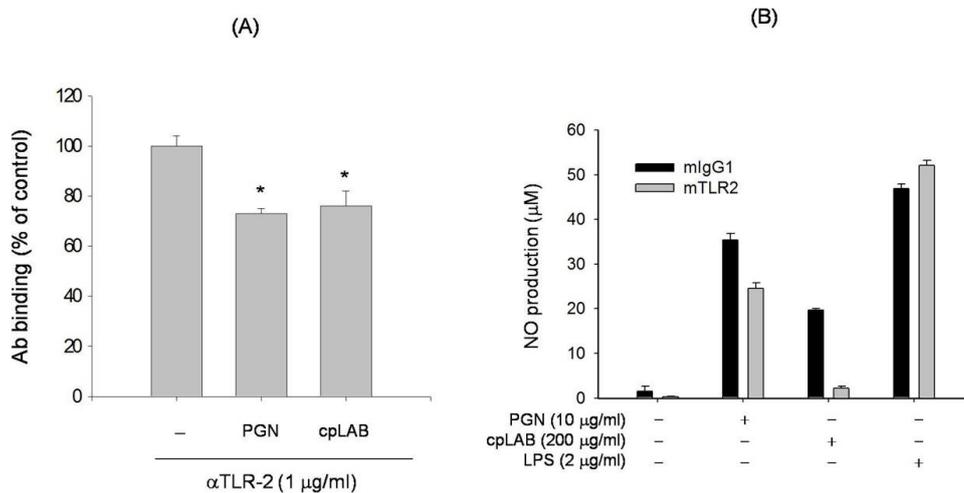


Fig. 4. The involvement of TLR-2 in cpLAB-induced NO production in RAW264.7 cells. (A) RAW264.7 cells (2×10^6 cells/ml), pretreated with cpLAB (100 μg/ml), were stained with antibody to TLR-2 (1 μg/ml) for 1 h. The extent of the antibody binding was determined by flow cytometric analysis, as described in Materials and Methods. (B) RAW264.7 cells (1×10^6 cells/ml) pretreated with anti-TLR-2 antibody (5 μg/ml) or isotype control IgG1 (5 μg/ml) for 2 h were stimulated by non-sonicated cpLAB for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. Data represent mean ± SEM of three independent observations performed in triplicate. The results show one experiment out of three. * $p < 0.05$ and ** $p < 0.01$ compared to control.

phosphorylation of p38 and JNK was not striking seen throughout the incubation of cpLAB, as similarly obtained in NO production assay with SB203580 and SP600125. The phosphorylation patterns of ERK observed during sonicated form treatment were slightly different from non-sonicated cpLAB case. Thus, sonicated sample, only triggered ERK phosphorylation from 5 min after treatment (Fig. 3C), whereas non-sonicated one remarkably increased from 30 min (Fig. 3B). How the difference in induction of phospho-ERK level between sonicated or non-sonicated forms was occurred is not yet clear in this study. Therefore, further experiments will be followed in terms of this point.

It has been known that the major immunogens from Gram (+) bacteria are PGN and lipoteichoic acid, ligands of TLR-2 (Gust *et al.*, 2007; Ryu *et al.*, 2009). Therefore, the role of TLR-2 in cpLAB-mediated macrophage responses was elucidated using flow cytometry with a specific monoclonal antibody to TLR-2. To know whether cpLAB binds to TLR-2, the binding effect of anti-TLR-2 antibody to the cells pretreated or post-treated with cpLAB was compared. As Fig. 4A shows, cpLAB pre-treatment significantly blocked the antibody binding up to 25%, suggesting that TLR-2 may be a target receptor for cpLAB, although the binding was not completely diminished. Interestingly, the NO production by cpLAB was strongly suppressed by the blocking antibody to TLR-2, as seen in the case of PGN, a ligand of TLR-2, unlike LPS and isotype control IgG1 treatment (Fig. 4B), suggesting a functional link between TLR-2 and cpLAB. However, cpLAB-mediated NO production was not affected by MTS510 (data not shown), a blocking antibody to TLR-4 (Kawasaki *et al.*, 2003), indicating that TLR-4 is not involved in cpLAB-induced macrophage responses.

In summary, we found that cpLAB is able to up-regulate NO production as well as iNOS expression from macrophages. The cpLAB-induced NO production seems to be managed by ERK pathway, since U0126 strongly suppressed NO production. cpLAB-mediated activation of macrophages may be required for the functional activation of TLR-2, but not TLR-4. Therefore, our data suggest that cpLAB may directly allow macrophages to immunostimulating potency via activation of TLR-2 and ERK.

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