***Lactobacillus plantarum* L67 glycoprotein protects against cadmium chloride toxicity in RAW 264.7 cells**

Sooyeon Song, Sejong Oh,¹ and Kye-Taek Lim¹

Division of Animal Science, Chonnam National University, 300 Yongbong-Dong, Gwang-ju 500-757, South Korea

ABSTRACT

The food and water we consume may be contaminated with a range of chemicals and heavy metals, such as lead, cadmium, arsenic, chromium, and mercury by accumulation through the food chain. Cadmium is known to be one of the major components in cigarette smoke and can cause lesions in many organs. Some lactobacilli can bind and remove heavy metals such as cadmium, lead, and copper. However, the mechanisms of cadmium toxicity and inhibition by probiotics are not clear. In this study, we demonstrated that glycoprotein (18 kDa) isolated from *Lactobacillus plantarum* L67 protected RAW 264.7 cells from expression of inflammation-related factors stimulated by cadmium chloride (100 μ M). Furthermore, we evaluated the cytotoxicity of cadmium using the MTT assay and intracellular Ca^{2+} using fluorescence, and assessed activities of activator protein kinase C (PKC- α), inducible nitric oxide synthase, activator protein (AP)-1, and mitogen-activated protein kinases using immunoblot. Our results indicated that glycoprotein isolated from *L. plantarum* L67 inhibited intracellular Ca^{2+} mobilization. It also significantly suppressed inflammatory factors such as AP-1 (c-Jun and c-Fos), mitogen-activated protein kinases (ERK, JNK, and p38), and inducible nitric oxide synthase. Our findings suggest that the 24-kDa glycoprotein isolated from *L. plantarum* L67 might be used as a food component for protection of inflammation caused by cadmium ion.

Key words: activator protein (AP)-1, cadmium, glycoprotein, *Lactobacillus plantarum*, mitogen-activated protein kinases (MAPK)

INTRODUCTION

The recent growth in human industrial activity has led to exponential increases in the amounts of heavy

metals released into the atmosphere, water, and soil (McConnell and Edwards, 2008). Cadmium (Cd) is a toxic heavy metal with a variety of adverse effects on the health of humans and animals. The risk of human exposure to heavy metal is increasing because of increasing Cd contamination of the food chain (Satarug et al., 2011). In recent years, many chronic Cd poisoning incidents have occurred due to inadequate protection from Cd-contaminated food and industrial material high-dose exposure (Fowler et al., 2011). Cadmium is present in almost all foods but the concentrations vary depending on the type of food and the level of environmental contamination (Saha et al., 2010). Food from plants generally contains higher concentrations of Cd than do meat, eggs, milk, dairy products, and fish (Buchauer, 1973; Olsson et al., 2002). Cadmium can cause lesions in different organs including kidney, testis, lung, liver, brain, bone, and blood. Inflammation, adenoma, and carcinoma are reportedly caused by exposure to Cd (Jarup et al., 1998; Nair et al., 2013).

The body's response to inflammation, adenoma, and carcinoma caused by Cd involves various factors. Intracellular Ca^{2+} has a role as secondary messenger in multiple signaling cascades (Batiza et al., 1996). Protein kinase C (PKC) is activated by intracellular Ca^{2+} . When PKC is activated, it is translocated from the cytosol to cellular compartments (Nakashima, 2002). It has been reported that PKC α is activated by a variety of stimuli such as physiological stresses. The activated PKC α subsequently transfers the signal to the mitogen-activated protein kinase (MAPK) pathways (Nakashima, 2002); MAPK is one of the important molecules involved in Cd-induced neurotoxicity (Kim et al., 2005; Chen et al., 2011; Thévenod and Lee, 2013). The MAPK are composed of a signaling cascade of serine-threonine kinases. They include extracellular signal-regulated kinase (ERK), p38, and c-Jun NH₂-terminal kinase (JNK). Observations suggest that MAPK contribute to inflammatory responses and neuronal death in neurodegenerative diseases, and that activated MAPK contribute to cell proliferation and carcinogenesis (Ito et al., 1998; Chang and Karin, 2001; Kim and Choi, 2010).

Received July 15, 2015.

Accepted September 7, 2015.

¹Corresponding authors: soh@chonnam.ac.kr and ktlim@chonnam.ac.kr

Activator protein (AP)-1 is a transcriptional factor, as well as a redox-sensitive oncogenic protein, and it controls the expression of cell growth mediators. It is a heterodimer consisting of the of Jun and Fos proteins (Kouzarides and Ziff, 1988), and is associated with malignant transformation, tumor promotion, and carcinogenic development. It regulates expression of inducible nitric oxide synthase (iNOS) pro-inflammatory factors (Lee and Lim, 2011).

Lactic acid bacteria have a long history as probiotics (Fernández et al., 2015). Several reports describe the various effects of *Lactobacillus* species including anti-tumor, anticoagulant, antiviral, immune regulator, and antiinflammatory, antidiabetic, and antioxidant or free radical scavenging activity (Hong et al., 2014; Song et al., 2015a,b). Additionally, studies have revealed that some *Lactobacillus*, such as *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus brevis*, can bind and remove heavy metals such as cadmium, lead, and copper in vitro (Halttunen et al., 2008; Mrvcic et al., 2009). We recently reported that membrane proteins (12 and 15 kDa) isolated from *L. plantarum* L67 induced apoptosis in human colorectal adenocarcinoma cells (Song et al., 2015b). Glycoproteins of some *Lactobacillus* have been known to have beneficial effects including regulating immature dendritic cells, modulating T-cell functions, and inhibiting the adherence of pathogens in intestines (Rhee and Park, 2001; Johnson-Henry et al., 2007; Konstantinov et al., 2008).

Therefore, the purpose of this study was to investigate the inhibitory effect of glycoprotein (18 kDa) isolated from *L. plantarum* L67 (L67 protein) in cadmium chloride-treated RAW 264.7 cells (a murine macrophage line). We also attempted to determine the possible mechanism of factors mediated with inflammation, cell proliferation, and cell cycle control. Toxicity of Cd, intracellular Ca^{2+} mobilization, and activity of PKC were measured using the MTT assay and a fluorescence method, respectively. The expression of MAPK and AP-1 (c-Jun and c-Fos) was evaluated using Western blotting. Finally, we demonstrated that *L. plantarum* L67 is a starter culture strain with a protective effect against Cd toxicity.

MATERIALS AND METHODS

Chemical

All plastic materials were purchased from Falcon Labware (Becton Dickinson, Franklin Lakes, NJ). Penicillin G and streptomycin were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco BRL (Grand

Island, NY). Other chemicals and reagents were of the highest analytical grade available.

Bacteria

Lactobacillus plantarum L67 (GenBank accession number: KR336551) strains from infant feces were selected according to Song et al. (2014). For identification of *L. plantarum* L67, the biochemical properties were first examined using an API 50CHL kit (bioMérieux, Marcy l'Etoile, France) and then by 16S rDNA sequencing. *Lactobacillus plantarum* L67 was grown in de Man, Rogosa, and Sharpe (MRS) broth (BD Difco, Le Pont de Claix, France) at 37°C for 18 h. The bacterial cells were separated by centrifugation at $3,000 \times g$ for 15 min at room temperature. Subsequently, the precipitated cells were washed twice with sterile saline (0.85% sodium chloride). One milliliter of 10% skim milk was added to the sediments and the suspensions were stored at -80°C until use.

Growth of *L. plantarum* L67 in Skim Milk and Preparation of Glycoprotein

Two hundred milliliters of skim milk broth was inoculated with *L. plantarum* L67 (1%) and incubated at 37°C. Cells were serially diluted with 1% peptone water and viable cells were counted every 3 h for 24 h.

For protein preparation, 10 mL of cultures were obtained after 18 h at 37°C. Pellets were collected by centrifugation ($12,000 \times g$, 4°C) and washed 3 times using sterile deionized water. The washed cells were resuspended in 10 g/L SDS solution and boiled for 10 min. The supernatants were harvested after centrifugation ($12,000 \times g$, 4°C, 15 min) and dialyzed against distilled water at 4°C. The supernatants were dried with a freeze-dryer and stored at -70°C . Protein concentrations were measured using the Bradford assay (Bradford, 1967). To confirm the results, SDS-PAGE was performed with the protein (100 mg/mL) containing 0.1% SDS, using a 14% polyacrylamide mini-gel and a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA) at 110 V and 30 mA. The L67 protein was confirmed by staining with Schiff's reagent (Neville and Glossmann, 1974).

Cell Culture and Cytotoxicity

Murine RAW 264.7 cells were incubated in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in an atmosphere containing 5% CO_2 at 37°C. The medium was renewed twice per

week. Cells (1×10^6 cells/mL) were distributed into 35-mm culture dishes or 96-well flat-bottomed plates. The final volumes were 2 mL/dish in the 35-mm culture dishes and 100 μ L/well in the 96-well flat-bottomed plates. The cytotoxicity of cadmium chloride and inhibitory effect of L67 total protein were determined by the MTT assay (Patton et al., 1989).

Detection of Intracellular Ca^{2+} Mobilization

Intracellular calcium was determined as described previously (Kimata et al., 1999). Briefly, cells were treated with L67 total protein (5, 10, 20, 50, and 100 μ g/mL) for 12 h. Cells were rinsed with a bath solution (140 mmol of NaCl, 5 mmol of KCl, 1 mmol CaCl_2 , 0.5 mmol of MgCl_2 , 10 mmol of glucose, 5.5 mmol of HEPES, pH 7.5) and then preincubated in bath solution containing 1 μ mol of Fura-2/AM (Molecular Probes, Eugene, OR) with 5% CO_2 at 37°C for 45 min with gentle shaking. Cells were treated with 100 μ M cadmium chloride for 3 h. Fluorescence intensity was measured at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm using a fluorescent microplate reader (Dual Scanning SpectraMax, Molecular Devices, Sunnyvale, CA).

Membrane, Cytosolic, and Whole-Protein Extract from RAW 264.7 Cells

The cells were rinsed twice with PBS after removing the medium and scraped in 300 μ L of buffer A [20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, and 2 mM EGTA] containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany). The cells were sonicated and centrifuged for 1 h at $10,000 \times g$ to sediment all membranes and the insoluble cytoskeletal components. The supernatant was designated as the cytosolic fraction. The membrane proteins in the pellet were extracted with buffer B (20 mM Tris-HCl, pH 7.5, 1% Nonidet P-40 (Sigma), 150 mM NaCl, 1 mM EGTA, and 1 mM EDTA) containing a protease inhibitor cocktail (Boehringer) on ice for 30 min and centrifuged for 15 min at $100,000 \times g$ at 4°C. The supernatant was saved as the detergent-soluble membrane fraction. Then, either nucleic protein extract for the immunoblotting of AP-1 (c-Jun and c-Fos) and proliferating cell nuclear antigen (PCNA) or whole cellular protein extract for the immunoblotting of MAPK (ERK, p38, and JNK) was isolated from RAW 264.7 cells as previously described (Oh and Lim, 2008). The amount of protein was measured via the method developed by Lowry et al. (1951) and cellular proteins were stored at -70°C before use.

Western Blot Analysis

Cellular proteins were separated on a 10% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a Mini-Protein II Electrophoresis Cell (Bio-Rad). After electrophoresis, the proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA). The membranes were subsequently incubated for 2 h at room temperature with primary antibodies (PKC α , ERK, phospho-ERK, JNK, phospho-JNK, and AP-1 (c-Fos and c-Jun) in Tris-buffered saline-Tween (TBS-T) solution. After washing 3 times with TBS-T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG (1:10,000; Cell Signaling Technology, Danvers, MA) in TBS-T solution. The resulting protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Bristol, UK). The results of the immunoblot assay were calculated as relative intensity using Scion imaging software (Scion Image Beta 4.02, Scion, Frederick, MD).

Statistical Analysis

All experiments were carried out in triplicate and data were expressed as the means $\pm$ standard errors ($n = 6$). The statistical significance of differences in results obtained under different experimental conditions was analyzed by 2-way ANOVA followed by Tukey's post hoc test for individual treatments.

RESULTS

Growth of *L. plantarum* L67 in Skim Milk and Isolation of Glycoprotein

As a first step to use *L. plantarum* L67 as starter culture strain, we constructed a growth curve for *L. plantarum* L67 in skim milk at 37°C. Figure 1 shows the growth curve of *L. plantarum* L67 in skim milk at 37°C. The *L. plantarum* L67 on the skim milk broth at 37°C reached 8.0 log cfu/mL after 6 h, and 8.7 log cfu/mL after 12 h. After 24 h, viable cells reached 8.3 log cfu/mL.

For the glycoprotein isolated from *L. plantarum* L67, the gel was stained with Schiff's reagent. Figure 2 indicates that the glycoprotein isolated from *L. plantarum* L67 had a molecular weight of 18 kDa.

Cytotoxicity of Cd and Anticytotoxicity Effect of L67 Protein

As shown in Figure 3, when the RAW 264.7 cells were treated with 100 μ M Cd for 3 h, the half-maximal

Figure 1. Growth curve of *Lactobacillus plantarum* L67 on skim milk. Two hundred milliliters of skim milk broth was inoculated with *L. plantarum* L67 (1%) and incubated at 37°C. The cells were serially diluted with 1% peptone water to count viable cells every 3 h for 24 h. Error bars indicate SE.

inhibitory concentration (IC_{50}) of RAW 264.7 cells was 100 μM . However, when the RAW 264.7 cells were treated with Cd (100 μM) after pretreatment with L67 protein (5, 10, 20, 50, and 100 g/mL), survivability increased significantly in a dose-dependent manner, compared with that without pretreatment with L67 protein. For example, the survivability of Cd-treated RAW 264.7 cells was increased by 64, 80, 88, 90, and 98% following pretreatment with 5, 10, 20, 50, and 100 $\mu g/mL$ of L67 protein in the presence of Cd (100 μM), respectively.

Inhibitory Effect of L67 Protein on Intracellular Ca^{2+} Level

We examined the involvement of intracellular Ca^{2+} level in Cd-treated RAW 264.7 cells. As shown in Figure 4, intracellular Ca^{2+} level increased by 1.15-fold when cells were treated with Cd compared with the control. However, with the L67 protein treatment, Cd induced an increase of intracellular Ca^{2+} in a concentration-dependent manner. Intracellular Ca^{2+} concentration underwent a 0.08-fold reduction following pretreatment with L67 protein (100 $\mu g/mL$) in the presence of Cd, compared with the Cd treatment without L67 pretreatment.

Inhibitory Effect of L67 Protein on Activity of PKC α in Cd-Treated RAW 264.7 Cells

As shown in Figure 5, when cells were treated with Cd, translocation of PKC α from the cytosol to the membrane was induced at 3 h after treatment. Treat-

ment with Cd (100 μM) resulted in a 0.23-fold reduction of band intensity of PKC α in the cytosolic extract, whereas intensity increased by 2.59-fold in the membrane extract, compared with the controls. However, when the cells were treated with Cd after pretreatment with L67 protein, PKC α translocation was inhibited in the Cd-treated RAW 264.7 cells in a concentration-dependent manner. Relative intensities of PKC α band in the membrane fraction underwent 0.37- and 0.54-fold reductions with a pretreatment of L67 at 50 and 100 $\mu g/mL$, respectively.

Figure 2. Identification of the L67 protein from *Lactobacillus plantarum*. Electrophoresis was performed on 14% acrylamide gel and the gel stained with Schiff's reagent for glycoprotein isolated from *L. plantarum* L67. M = marker; L67 = glycoprotein isolated from *L. plantarum* L67.

Figure 3. Cytotoxicity of Cd and anticytotoxicity effect of L67 protein on Cd (100 μ M)-treated RAW 264.7 cells. The cytotoxicity of cadmium chloride measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was dose-dependent (A), as was anticytotoxicity effect following pretreatment with L67 protein (5–100 μ g/mL) (B). All data represent means $\pm$ SE from triplicates. #Significant difference between the treatment and control groups ($P < 0.05$); * and **significant difference between Cd treatment and L67 protein pretreatment in the presence of Cd (* $P < 0.05$, ** $P < 0.01$). Cont = control.

Inhibitory Effect of L67 Protein on Expression of iNOS in Cd-Treated RAW 264.7 Cells

As shown in Figure 5, pretreatment with L67 protein inhibited expression of iNOS in Cd-stimulated RAW 264.7 cells. For instance, in cells that were treated with Cd, expression of iNOS was significantly increased compared with the control. However, when cells were treated with a pretreatment of L67 protein (5–100 μ g/mL), activity of iNOS decreased significantly in a dose-dependent manner (Figure 6). The relative intensities of iNOS were increased 2.27-fold after treatment with Cd compared with those in the control. However, such activity underwent a 0.88-fold reduction for iNOS after the treatment with Cd and pretreatment of L67 protein (100 μ g/mL) compared with Cd treatment alone.

Inhibitory Effect of L67 Protein on Phosphorylation of ERK, JNK, and p38 in Cd-Treated RAW 264.7 Cells

To determine whether pretreatment with L67 protein inhibits ERK and JNK phosphorylation in the Cd-treated RAW 264.7 cells, we assessed ERK and JNK phosphorylation after treatment with Cd for 3 h. Activities of both JNK and ERK were increased by treatment with 100 μ M Cd compared with the controls (Figure 7). Relative band intensities of ERK and JNK underwent 10- and 21-fold increases, respectively, compared with treatment with Cd treatment alone. However, when the cells were treated with Cd after pretreatment of L67 protein (5, 10, 20, 50, and 100 μ g/mL), the relative band intensities were reduced in a dose-dependent manner. When the cells were treated with Cd (100 μ M) after pretreatment with L67 protein (100 μ g/mL), the band intensities of ERK and JNK were significantly reduced by 7- and 21-fold, respectively, compared with the Cd treatment alone.

Inhibitory Effect of L67 Protein on Transcriptional Factor AP-1 in Cd-Treated RAW 264.7 Cells

We evaluated the effects of L67 protein on activation of AP-1 (c-Jun and c-Fos) using Western blotting analysis. When the cells were treated with 100 μ M Cd for 3 h, AP-1 activity was markedly increased compared with the control. However, when the cells were pretreated with L67 protein (50 and 100 μ g/mL)

Figure 4. Inhibitory effect of L67 protein from *Lactobacillus plantarum* on mobilization of intracellular Ca²⁺ in Cd-treated RAW 264.7 cells. The cells were treated with 100 μ M Cd without or with pretreatment with L67 protein (5, 10, 20, 50, and 100 μ g/mL). Changes in intracellular Ca²⁺ concentration were monitored with Fura-2/AM, as described in the Materials and Methods. Data were expressed as the absolute intensity of Ca²⁺ fluorescence and are shown as means $\pm$ SE from triplicates ($n = 9$). #Significant difference between the treatment and control groups ($P < 0.05$); * and **significant difference between Cd treatment and L67 protein pretreatment in the presence of Cd (* $P < 0.05$, ** $P < 0.01$). Cont = control.

Figure 5. Inhibitory effect of L67 protein from *Lactobacillus plantarum* on the activity of protein kinase C (PKC) in Cd-treated RAW 264.7 cells. The cells were treated with Cd (100 μ M) alone or pretreated with L67 protein (5, 10, 20, 50, and 100 μ g/mL) and Cd for 3 h. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02; Scion, Frederick, MD). Data were represented as absolute intensity and expressed as means $\pm$ SE from triplicates ($n = 9$). [#]Significant difference between treatments and controls ($P < 0.05$); * and **significant difference between Cd treatment and L67 protein pretreatment in the presence of Cd (* $P < 0.05$, ** $P < 0.01$). Cont = control.

and treated with 100 μ M Cd, the activity of AP-1 was significantly reduced in a concentration-dependent manner compared with the Cd treatment alone. The relative intensities of AP-1 (c-Jun and c-Fos) were augmented 10-fold and 76-fold following 100 μ M Cd treatment alone compared with the controls. However, when the cells were pretreated with L67 protein (100 μ g/mL) before treatment with Cd, the activities of AP-1 (c-Jun and c-Fos) were diminished by 6-fold and 65-fold, respectively, compared with Cd alone (Figure 8). However, when the cells were pretreated with L67 protein (100 μ g/mL) in the presence of Cd, the activities of AP-1 (c-Jun and c-Fos) were reduced on treatment with L67 protein (5, 10, 20, 50, and 100 μ g/mL) in a dose-dependent manner.

DISCUSSION

Cadmium, a well-known carcinogen and immunotoxin that is commonly found in cigarette smoke and indus-

Figure 6. Inhibitory effect of L67 protein from *Lactobacillus plantarum* on expression of inducible nitric oxide synthase (iNOS) in Cd-treated RAW 264.7 cells. The cells were treated with Cd (100 μ M) alone or pretreated with the L67 protein (5, 10, 20, 50, and 100 μ g/mL) and Cd for 3 h. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02; Scion, Frederick, MD). Data were represented as absolute intensity and expressed as means $\pm$ SE from triplicates ($n = 9$). [#]Significant difference between treatments and controls ($P < 0.05$); *significant difference between Cd treatment and L67 protein pretreatment in the presence of Cd ($P < 0.05$). Cont = control.

trial effluent, poses a toxicity risk for humans because of its presence in the food chain. In this investigation, we attempted to determine whether L67 protein from *L. plantarum* would protect RAW 264.7 cells from expression of inflammation-related factors stimulated by cadmium chloride. As a first step to use *L. plantarum* L67 as the starter culture strain, we constructed a growth curve for *L. plantarum* L67 in skim milk at 37°C. This strain can be used as starter culture and has probiotic potential. To determine the cytotoxicity of Cd in RAW 264.7 cells, we used the MTT assay. The results indicated an IC_{50} of 100 μ M after a 3-h treatment. We also determined survivability of RAW 264.7 cells pretreated with L67 protein at different concentrations (5–100 μ g/mL). The results showed that pretreatment with L67 protein improved the survivability of Cd-treated RAW 264.7 cells (Figure 3).

Cadmium can disrupt signaling cascades that lead to a variety of toxic effects because of the similarities between Cd^{2+} and calcium ion (Ca^{2+}). Many studies have reported how Cd can effect changes in calcium signaling pathways and their resultant toxic effects. Differences in calcium signal duration and amplitude and subcellular location of the stimulus can affect processes such as cell division, cell motility, muscle contraction, meta-

Figure 7. Inhibitory effect of L67 protein from *Lactobacillus plantarum* on phosphorylation (Phospho- or P) of extracellular signal-regulated kinase (ERK) and c-Jun NH₂-terminal kinase (JNK) in Cd-treated RAW 264.7 cells. The cells were treated with Cd (100 μM) for 3 h alone or pretreated with L67 protein (5, 10, 20, 50, and 100 μg/mL). The relative intensities of bands were calculated with Scion Imaging Software (Scion Image Beta 4.02; Scion, Frederick, MD). Data were represented as absolute intensities and expressed as means ± SE from triplicates (n = 9). [#]Significant difference between the treatment and control groups ($P < 0.05$); ^{*}significant difference between Cd treatment and L67 protein in the presence of Cd ($P < 0.05$). Cont = control.

bolic activity, and apoptosis (Cheung, 1988; Roderick and Cook, 2008). This study showed that intracellular Ca²⁺ was released by treatment with Cd alone in RAW 264.7 cells, whereas L67 protein inhibited the amounts of Ca²⁺ in the presence of Cd (Figure 4). Thus, changes in calcium signaling caused by Cd were normalized or inhibited by pretreatment with L67 protein. In addition, the change in Ca²⁺ amount was closely related to mobilization of PKC (activation) from the cytosol to inside the cell membrane, because PKC activation depends on Ca²⁺. In this study, the L67 protein suppressed the translocation (activation) of PKCα from the cytosol to the cell membrane in Cd-treated RAW 264.7 cells (Figure 5). Activation of PKC plays a critical role in cell survival, proliferation, and tumor promotion (Zheng et al., 2010). The activation of the ERK1/2 and JNK pathways is mediated by PKC isoforms (Dobkin-Bekman et al., 2010).

Inducible NOS plays important roles as a regulator of inflammatory reactions. Activity of iNOS and overproduction of nitric oxide (NO) are related to increased risk of many diseases (Chung et al., 2007). Inducible NOS is an inflammation and pro-tumor mediator, but it also causes the overproduction of nitric oxide (Chung et al., 2007). Low levels of NO usually regulate many physiological processes, whereas over-

production of NO, as a free radical, increases oxidative stress and participates in pathogenesis (Lee and Lim, 2011). Synthesis of iNOS is significantly induced in macrophages from cadmium-treated mice (Ramirez and Gimenez, 2003). In the current study, iNOS activity was increased by Cd treatment, whereas L67 protein inhibited iNOS activity in Cd-stimulated RAW 264.7 cells. (Figure 6).

Mitogen-activated protein kinases are essential components of intracellular signal transduction and are activated by phosphorylation in response to a variety of extracellular stimuli including growth factors, cytokines, and environmental stress (Ito et al., 1998; Chang and Karin, 2001; Kim and Choi, 2010). Cadmium toxicity upregulates several genes in MAPK pathways. The activation of MAPK can play an important role in Cd-induced cellular apoptosis (Tzirogiannis et al., 2003; Scholz-Ahrens et al., 2007; Kruger et al., 2009). Zhai et al. (2014) showed that co-treatment with Cd and *Lactobacillus plantarum* reverses some of these altered gene expressions in the MAPK pathway, indicating that the antioxidative stress effect of *Lactobacillus plantarum* may be related to regulation of these signal transduction pathways, which in turn decreases production of reactive oxygen species and prevents Cd-induced cellular apoptosis (Zhai et al., 2014). Wang et al. (2014)

found that ERK, p38, and JNK were activated by Cd in neuronal cells *in vitro* and *in vivo*, and that activation of ERK or JNK promoted cell death, whereas activation of p38 inhibited cell death. As shown in Figure 7, our findings also demonstrated that phosphorylated ERK and JNK were strongly expressed by treatment with Cd and inhibited by pretreatment with L67 protein. The activation of the ERK and JNK pathways, which results in increased expression of c-Fos, c-Jun, or both, has been reported in some chemically treated cells (Chang et al., 2009). Moreover, ERK transfers signals to the nuclei to induce proliferation and differentiation, by stimulating the transcription factors that induce expression of c-Fos and other compounds (Lopez-Illasaca, 1998).

Activator protein-1 in the nucleus is influenced by the cellular redox status and is thought to play important roles in proinflammatory and carcinogenic events (Lee and Lim, 2011). It was reported to regulate expression of cyclooxygenase-2 (COX-2) and iNOS proinflammatory factors (Lee and Lim, 2011). In addition, AP-1 activation is induced at multiple levels by MAPK activation involving the major pathways of ERK and

JNK (Shen et al., 2006). In many studies, Cd induced overexpression of c-Fos and c-Jun transcription factors (Bertin and Averbeck, 2006; Yang et al., 2007). In the current study, AP-1 was activated in response to Cd but inhibited by pretreatment with L67 protein in the presence of Cd (Figure 8).

Based on the experimental findings, we made the following assumptions. The possible mechanism of cell proliferation inhibition by L67 protein is that the extract first inhibits calcium mobilization and depresses translocation of PKC. Subsequently, the signals ERK, JNK, and AP-1, which are located downstream of PKC, were not activated by L67 protein.

Two possible mechanisms might explain protection from Cd-induced carcinogenesis of RAW 264.7 cells by L67 protein. First, L67 protein might have negatively charged AA residues that stimulate survival signal or increase bioactivity of RAW 264.7 cells. Negatively charged AA of L67 have high polarity and high protein moiety, which cause the protein to interact with the receptor on the membrane of RAW 264.7 cells. The result of this interaction is stimulation of signal-related kinases in the intracellular plasm. Then, the stimulated

Figure 8. Inhibitory effect of L67 protein from *Lactobacillus plantarum* on the transcriptional factor activator protein (AP-1; c-Jun and c-Fos) in Cd-treated RAW 264.7 cells. The cells were treated with Cd (100 μ M) for 3 h in the absence or pretreatment of L67 protein (5, 10, 20, 50, and 100 μ g/mL). The relative intensities of bands were calculated with Scion Imaging Software (Scion Image Beta 4.02; Scion, Frederick, MD). Data were represented as absolute intensities and expressed as means $\pm$ SE from triplicates ($n = 9$). [#]Significant difference between the treatment and control groups ($P < 0.05$); ^{*}significant difference between Cd treatment and L67 protein in the presence of Cd ($P < 0.05$). Cont = control.

kinase transfers the signal to survival or bioactivity. Second, the L67 protein interacts with the positive divalent cation of cadmium and then builds a complex compound before transit to the cell membrane.

In conclusion, we showed that pretreatment with L67 protein inhibits cytotoxicity and intracellular Ca^{2+} mobilization following exposure of RAW 264.7 cells to cadmium chloride. Pretreatment with L67 protein also significantly suppressed the expression of AP-1 (C-fos and C-jun) and MAPK (ERK and JNK). These findings suggest that L67 protein might protect against inflammation caused by the cadmium ions naturally present in food. In addition, *L. plantarum* L67 can be used as a starter culture strain with a potential protective effect against cadmium toxicity.

ACKNOWLEDGMENTS

This work was completely financed by iPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries; Republic of Korea) in 2014. The authors declare that they have no conflicts of interest.

REFERENCES

- Batiza, A. F., T. Schulz, and P. H. Masson. 1996. Yeast respond to hypotonic shock with a calcium pulse. *J. Biol. Chem.* 271:23357–23362.
- Bertin, G., and D. Averbeck. 2006. Cadmium: Cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie* 88:1549–1559.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Buchauer, M. J. 1973. Contamination of soil and vegetation near a zinc smelter by zinc, cadmium, copper, and lead. *Environ. Sci. Technol.* 7:121–135.
- Chang, C. H., F. Y. Yu, L. T. Wang, Y. S. Lin, and B. H. Liu. 2009. Activation of ERK and JNK signaling pathways by mycotoxin citrinin in human cells. *Toxicol. Appl. Pharmacol.* 237:281–287.
- Chang, L., and M. Karin. 2001. Mammalian MAP kinase signalling cascades. *Nature* 410:37–40.
- Chen, S., Y. Xu, B. Xu, M. Guo, Z. Zhang, L. Liu, H. Ma, Z. Chen, Y. Luo, S. Huang, and L. Chen. 2011. CaMKII is involved in cadmium activation of MAPK and mTOR pathways leading to neuronal cell death. *J. Neurochem.* 119:1108–1118.
- Cheung, W. Y. 1988. Calmodulin and its activation by cadmium ion. *Ann. N. Y. Acad. Sci.* 522:74–87.
- Chung, W. Y., J. H. Park, M. J. Kim, H. O. Kim, J. K. Hwang, S. K. Lee, and K. K. Park. 2007. Xanthorrhizol inhibits 12-O-tetradecanoylphorbol-13-acetate-induced acute inflammation and two-stage mouse skin carcinogenesis by blocking the expression of ornithine decarboxylase, cyclooxygenase-2 and inducible nitric oxide synthase through mitogen-activated protein kinases and/or the nuclear factor-kappa B. *Carcinogenesis* 28:1224–1231.
- Dobkin-Bekman, M., L. Rahamin Ben-Navi, B. Shterental, L. Sviridov, F. Przedecki, M. Naidich-Exler, C. Brodie, R. Seger, and Z. Naor. 2010. Differential role of PKC isoforms in GnRH and phorbol 12-myristate 13-acetate activation of extracellular signal-regulated kinase and Jun N-terminal kinase. *Endocrinology* 151:4894–4907.
- Fernández, M., J. A. Hudson, R. Korpela, and C. G. de Los Reyes-Gavilán. 2015. Impact on human health of microorganisms present in fermented dairy products: An overview. *BioMed Res. Int.* 2015:412714.
- Fowler, B. A., G. F. Nordberg, M. Nordberg, and L. Friberg. 2011. Chapter 23: Cadmium. Pages 446–470 in *Handbook on the Toxicology of Metals*. 3rd ed. G. F. Nordberg, B. A. Fowler, M. Nordberg, and L. Friberg, ed. Academic Press, Burlington, MA.
- Halttunen, T., M. C. Collado, H. El-Nezami, J. Meriluoto, and S. Salminen. 2008. Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution. *Lett. Appl. Microbiol.* 46:160–165.
- Hong, Y. F., H. Kim, H. R. Kim, M. G. Gim, and D. K. Chung. 2014. Different immune regulatory potential of *Lactobacillus plantarum* and *Lactobacillus sakei* isolated from Kimchi. *J. Microbiol. Biotechnol.* 24:1629–1635.
- Ito, Y., Y. Sasaki, M. Horimoto, S. Wada, Y. Tanaka, A. Kasahara, T. Ueki, T. Hirano, H. Yamamoto, J. Fujimoto, E. Okamoto, N. Hayashi, and M. Hori. 1998. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. *Hepatology* 27:951–958.
- Järup, L., M. Berglund, C. G. Elinder, G. Nordberg, and M. Vahter. 1998. Health effects of cadmium exposure—A review of the literature and a risk estimate. *Scand. J. Work Environ. Health* 24(Suppl. 1):1–51.
- Johnson-Henry, K. C., K. E. Hagen, M. Gordonpour, T. A. Tompkins, and P. M. Sherman. 2007. Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. *Cell. Microbiol.* 9:356–367.
- Kim, E. K., and E. J. Choi. 2010. Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta* 1802:396–405.
- Kim, S.D., C. K. Moon, S. Y. Eun, P. D. Ryu, and S. A. Jo. 2005. Identification of ASK1, MKK4, JNK, c-Jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis. *Biochem. Biophys. Res. Commun.* 328:326–334.
- Kimata, M., M. Shichijo, T. Miura, I. Serizawa, N. Inagawa, and H. Nagai. 1999. Ca^{2+} and protein kinase C signaling for histamine and sulfidoleukotrienes released from human cultured mast cells. *Biochem. Biophys. Res. Commun.* 257:895–900.
- Konstantinov, S. R., H. Smidt, W. M. de Vos, S. C. Bruijns, S. K. Singh, F. Valence, D. Molle, S. Lortal, E. Altermann, T. R. Klaenhammer, and Y. van Kooyk. 2008. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc. Natl. Acad. Sci. USA* 105:19474–19479.
- Kouzarides, T., and E. Ziff. 1988. The role of the leucine zipper in the fos-jun interaction. *Nature* 336:646–651.
- Kruger, M. C., A. Fear, W.-H. Chua, G. G. Plimmer, and L. M. Scholium. 2009. The effect of *Lactobacillus rhamnosus* HN001 on mineral absorption and bone health in growing male and ovariectomized female rats. *Dairy Sci. Technol.* 89:219–231.
- Lee, J., and K. T. Lim. 2011. Preventive effect of phytylglycoprotein (27 kDa) on inflammatory factors at liver injury in cadmium chloride-exposed ICR mice. *J. Cell. Biochem.* 112:694–703.
- Lopez-Illasaca, M. 1998. Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem. Pharmacol.* 56:269–277.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
- McConnell, J. R., and R. Edwards. 2008. Coal burning leaves toxic heavy metal legacy in the Arctic. *Proc. Natl. Acad. Sci. USA* 105:12140–12144.
- Mrvic, J., D. Stanzer, V. Bacun-Druzina, and V. Stehlik-Tomas. 2009. Copper binding by lactic acid bacteria (LAB). *Biosci. Microflora* 28:1–6.
- Nair, A. R., O. Degheselle, K. Smeets, E. Van Kerkhove, and A. Cuypers. 2013. Cadmium-induced pathologies: Where is the oxidative balance lost (or not)? *Int. J. Mol. Sci.* 14:6116–6143.
- Nakashima, S. 2002. Protein kinase C alpha (PKC alpha): regulation and biological function. *J. Biochem.* 132:669–675.

- Neville, D. M. Jr., and H. Glossmann. 1974. Molecular weight determination of membrane protein and glycoprotein subunits by discontinuous gel electrophoresis in dodecyl sulfate. *Methods Enzymol.* 32:92–102.
- Oh, P. S., and K. T. Lim. 2008. Blocking of intracellular ROS production by phytylglycoprotein (30 kDa) causes anti-proliferation in bisphenol A stimulated Chang liver cells. *J. Appl. Toxicol.* 28:749–758.
- Olsson, I. M., I. Bensryd, T. Lundh, H. Ottosson, S. Skerfving, and A. Oskarsson. 2002. Cadmium in blood and urine: Impact of sex, age, dietary intake, iron status, and former smoking—Association of renal effects. *Environ. Health Perspect.* 110:1185–1190.
- Patton, W. F., M. R. Dhanak, and B. S. Jacobson. 1989. Differential partitioning of plasma membrane proteins into the triton X-100-insoluble cytoskeleton fraction during concanavalin A-induced receptor redistribution. *J. Cell Sci.* 92:85–91.
- Ramirez, D. C., and M. S. Gimenez. 2003. Induction of redox changes, inducible nitric oxide synthase and cyclooxygenase-2 by chronic cadmium exposure in mouse peritoneal macrophages. *Toxicol. Lett.* 145:121–132.
- Rhee, C. H., and H. D. Park. 2001. Three glycoproteins with antimutagenic activity identified in *Lactobacillus plantarum* KLAB21. *Appl. Environ. Microbiol.* 67:3445–3449.
- Roderick, H. L., and S. J. Cook. 2008. Ca²⁺ signalling checkpoints in cancer: Remodelling Ca²⁺ for cancer cell proliferation and survival. *Nat. Rev. Cancer* 8:361–375.
- Saha, J. K., N. R. Panwar, and M. V. Singh. 2010. Determination of lead and cadmium concentration limits in agricultural soil and municipal solid waste compost through an approach of zero tolerance to food contamination. *Environ. Monit. Assess.* 168:397–406.
- Satarug, S., S. H. Garrett, M. A. Sens, and D. A. Sens. 2011. Cadmium, environmental exposure, and health outcomes. *Cien. Saude Colet.* 16:2587–2602. <http://dx.doi.org/10.1590/S1413-81232011000500029>.
- Scholz-Ahrens, K. E., P. Ade, B. Marten, P. Weber, W. Timm, Y. Avarsigmail, C. C. Glüer, and J. Schrezenmeir. 2007. Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. *J. Nutr.* 137:838S–846S.
- Shen, F., X. Fan, B. Liu, X. Jia, H. Du, B. You, M. Ye, C. Huang, and X. Shi. 2006. Overexpression of cyclin D1-CDK4 in silica-induced transformed cells is due to activation of ERKs, JNKs/AP-1 pathway. *Toxicol. Lett.* 160:185–195.
- Song, M., B. Yun, J. K. Moon, D. J. Park, K. Lim, and S. Oh. 2015a. Characterization of selected *Lactobacillus* strains for use as probiotics. *Korean J. Food Sci. Anim. Resour.* 35:551–556.
- Song, S., D. W. Bae, K. Lim, M. W. Griffiths, and S. Oh. 2014. Cold stress improves the ability of *Lactobacillus plantarum* L67 to survive freezing. *Int. J. Food Microbiol.* 191:135–143.
- Song, S., S. Oh, and K. T. Lim. 2015b. The proteins (12 and 15 kDa) isolated from heat-killed *Lactobacillus plantarum* L67 induces apoptosis in HT-29 cells. *Cell Biochem. Funct.* 33:89–96.
- Thévenod, F., and W. K. Lee. 2013. Cadmium and cellular signaling cascades: Interactions between cell death and survival pathways. *Arch. Toxicol.* 87:1743–1786.
- Tzirogiannis, K. N., G. I. Panoutsopoulos, M. D. Demonakou, R. I. Hereti, K. N. Alexandropoulou, A. C. Basayannis, and M. G. Mykoniatis. 2003. Timecourse of cadmium-induced acute hepatotoxicity in the rat liver: The role of apoptosis. *Arch. Toxicol.* 77:694–701.
- Wang, B., J. L. Xiao, Y. H. Ling, X. J. Meng, B. Wu, X. Y. Yang, and F. Zou. 2014. BNIP3 upregulation by ERK and JNK mediates cadmium-induced necrosis in neuronal cells. *Toxicol. Sci.* 140:393–402.
- Yang, Z., S. Yang, S. Y. Qian, J. S. Hong, M. B. Kadiiska, R. W. Tennant, M. P. Waalkes, and J. Liu. 2007. Cadmium-induced toxicity in rat primary mid-brain neuroglia cultures: Role of oxidative stress from microglia. *Toxicol. Sci.* 98:488–494.
- Zhai, Q., G. Wang, J. Zhao, X. Liu, A. Narbad, Y. Q. Chen, H. Zhang, F. Tian, and W. Chen. 2014. Protective effects of *Lactobacillus plantarum* CCFM8610 against chronic cadmium toxicity in mice indicate routes of protection besides intestinal sequestration. *Appl. Environ. Microbiol.* 80:4063–4071.
- Zheng, L. P., D. L. Zhang, J. Huang, L. Q. Xu, A. X. Xu, X. Y. Du, D. F. Tang, and Y. H. Zheng. 2010. Proto-oncogene c-erbB2 initiates rat primordial follicle growth via PKC and MAPK pathways. *Reprod. Biol. Endocrinol.* 8:66.