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Antiinflammatory Effect of Lactic Acid Bacteria: Inhibition of Cyclooxygenase-2 by Suppressing Nuclear Factor-κB in Raw264.7 Macrophage Cells

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***Lactobacillus casei* 3260 (*L. casei* 3260) was evaluated in relation to the inflammatory response mediated by lipopolysaccharide (LPS)-induced nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2) expression in Raw264.7 macrophage cells. The treatment of Raw264.7 cells with *L. casei* 3260 significantly inhibited the secretion of tumor necrosis factor-α (TNF-α) and prostaglandins E_2 (PGE_2), followed by suppression of COX-2. To clarify the molecular mechanism, the inhibitory effect of *L. casei* 3260 on the NF-κB signaling pathway was examined based on the luciferase reporter activity. Although the treatment of Raw264.7 cells with *L. casei* 3260 did not affect the transcriptional activity of NF-κB, it did inhibit NF-κB activation, as determined by the cytosolic p65 release and degradation of I-κBα. Therefore, these findings suggest that the suppression of COX-2 through inhibiting the NF-κB activation by LPS may be associated with the antiinflammatory effects of *L. casei* 3260 on Raw264.7 cells.**

Keywords: *L. casei* 3260, NF-κB, COX-2, TNF-α, Raw264.7 cells

Prostaglandins E_2 (PGE_2) generated from arachidonic acid through the cyclooxygenase (COX) pathway is a major mediator in the regulation of inflammation and the immune function [23]. Thus, the interplay between PGE_2 and other local factors, including inflammatory cytokines, is likely to influence the outcome of inflammatory and immune responses. COX, which exists as two isoforms, is the rate-limiting enzyme in prostaglandin production. These isoforms (namely, constitutive COX-1 and inducible COX-2) originate from two distinct genes, yet are structurally conserved [7, 18]. COX-1 serves as a constitutive enzyme responsible for prostaglandin synthesis, which is essential for fluid and electrolyte homeostasis, gastric acid secretion, and platelet aggregation, whereas COX-2 is induced by inflammatory stimuli such as TNF-α and lipopolysaccharide (LPS) [6].

Extensive research over the last few years has shown that most inflammatory agents mediate their effects through the activation of nuclear factor κB (NF-κB), whereas most antiinflammatory agents suppress NF-κB activation. NF-κB is present in a resting state in all cells in the cytoplasm, and thus the above-mentioned events are only generated when NF-κB is activated and translocated into the nucleus. Under resting conditions, NF-κB consists of a heterotrimer of p50, p65, and I-κB in the cytoplasm. The phosphorylation, ubiquitination, and degradation of I-κB lead to the release of the p50-p65 heterodimer, which then translocates into the nucleus, binds its specific 10-base-pair consensus site, and regulates the genetic expression of key proinflammatory cytokines [8, 9]. The activation of NF-κB has recently been reported to participate in the transcriptional activation of the COX-2 gene [21, 16, 4]. Furthermore, LPS-induced activation of the COX-2 gene has been shown to be mediated by the inhibitor κB (I-κB) kinase (IKK), which specifically catalyzes I-κB phosphorylation, followed by its degradation and subsequent NF-κB nuclear translocation [9].

Various beneficial effects on human health have been attributed to probiotic bacteria, mainly on the intestinal level [1, 3, 20]. As a result, there is convergent evidence suggesting that some probiotics may have antiinflammatory properties. For example, it has been reported that gastric inflammation associated with a *Helicobacter pylori* infection was improved by supplementing with probiotic bacteria [11]. It has also been shown that *Lactobacillus rhamnosus* GG decreases TNF-α production by macrophages *in vitro* [19]. In addition, a recent study reported that treatment of

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HT-29 colon cancer cells with lactic acid bacteria or cultured media leads to the inhibition of NF-κB nuclear translocation and inflammatory cytokine production in a strain-dependent manner [17]. Therefore, this directly implies that other lactic acid bacteria may also be able to regulate the inflammatory response mediated by NF-κB activation. *L. casei* 3260, isolated from milk products, has been demonstrated to exert antioxidative activity by removing metal ions, and to eliminate toxic materials, such as AFB_1, by binding them to surface molecules, especially carbohydrate components, thereby suggesting that *L. casei* 3260 may qualify as a probiotic. Accordingly, the purpose of this study was to investigate the antiinflammatory effect of *L. casei* 3260 on Raw264.7 macrophage cells and the involvement of COX-2 suppression mediated by NF-κB inactivation.

Materials and Methods

Bacterial Strains

L. casei 3260 was obtained from the frozen stock culture collection of the Korean Collection for Type Cultures and grown at 37°C in deMan-Rogosa-Sharpe (MRS) broth (Oxoid, Hampshire, United Kingdom) for 72 h under anaerobic conditions. The *L. casei* 3260 was then serially transferred at least three times prior to use in the present study. The bacterial counts were determined by flow cytometry using a FACS Calibur equipped with an air-cooled 488-nm argon-ion laser at 15 mV, whereas the direct counts were enumerated using Fluoresbrite beads (2.0 μm; Polysciences Inc., Warrington, PA, U.S.A.) as an internal calibration. The viability of the bacterial population was assessed using SYTOX green nucleic acid stain (Molecular Probes Inc., Eugene, OR, U.S.A.) at 1 μM/10^6–10^7 bacteria to detect nonviable bacteria. A 525-nm band pass filter was used to collect green SYTOX emission. To support precise bacterial counts, the colony forming unit (CFU) was determined by plating 10-fold serial dilutions of the bacterial suspension on MRS agar plates. The strain was adjusted to a 1×10^9 CFU/ml for treatment in all the experiments.

Preparation of Raw264.7 Macrophage Cells

The Raw264.7 macrophage cell line (ATCC HTB37) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and maintained in minimal essential media (MEM; BioWhitaker, Walkersville, MD, U.S.A.) supplemented with 10% heat-inactivated (55°C, 30 min) fetal bovine serum (FBS; BioWhitaker, Walkersville, MD, U.S.A.), 2 mM L-glutamine (Sigma, St. Louis, MO, U.S.A.), and 100 U/ml penicillin and 100 mg/ml streptomycin (BioWhitaker, Walkersville, MD, U.S.A.) at 37°C in an atmosphere of 5% CO_2. Prior to treatment with the bacteria and 1 μg/ml LPS, the Raw264.7 cells were incubated in serum-free MEM for 17 h. In all the experiments, the cells were grown to 80–90% confluency and subjected to no more than 20 cell passages.

Cell Viability Assay

The number of viable cells was determined by the ability of the mitochondria to convert MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] into formazan dye. The Raw264.7 cells were cultured in a 96-well plate at a density of 1×10^4 cells/well. After 24 h of incubation with different numbers of bacteria, the medium was removed, 50 μg/ml of MTT in Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) added to each well, and the incubation continued for a further 4 h in a humidified atmosphere at 37°C, 5% CO_2. The MTT was then removed, the cells lysed with 100 μl of DMSO, and the absorbance measured at 570 nm and a reference wavelength of 665 nm on a microplate reader (BioTek, Winooski, VT, U.S.A.).

Measurement of TNF-α and PGE_2 from Raw264.7 Cells

The cells were cultured in 24-well plates at a density of 2×10^5 cells/well and stimulated with 1 μg/ml of LPS with or without *L. casei* 3260. The level of TNF-α and PGE_2 in the cultured medium of the Raw264.7 cells was then determined by sandwich ELISA. The anti-TNF-α and anti-PGE_2 monoclonal antibodies, biotinylated polyclonal antibody, and mouse TNF-α and PGE_2 standard materials were all obtained from R&D Systems (Minneapolis, MN, U.S.A.).

Western Blot Analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to the method in a previous report with a slight modification [15]. Briefly, after 24 h of incubation with *L. casei* 3260, the cells (2×10^6 cells/plate) were lysed in a buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM sodium chloride, 0.2% SDS, 1% NP-40, 5 mM sodium fluoride, and a protein inhibitor cocktail (Roche, Penzberg, Germany). The cell lysates were then centrifuged at 12,000 ×*g* for 30 min to remove any debris, fractionated by 12% gel electrophoresis, and electrophoretically transferred to nitrocellulose paper that had previously been incubated with polyclonal anti-COX-2 (1:200; Santa Cruz, CA, U.S.A.), monoclonal anti-I-κBα (1:200; Cell Signaling Technology, Beverly, MA, U.S.A.), monoclonal anti-phospho-p65 (1:200; Cell Signaling Technology, Beverly, MA, U.S.A.) or monoclonal anti-actin (1:100; Santa Cruz, CA, U.S.A.) antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (1:5,000; Santa Cruz, CA, U.S.A.). Finally, the papers were developed using an ECL chemiluminescence detection kit (Amersham, Piscataway, NJ, U.S.A.). Scanning densitometry was performed using an Image Scan & Analysis System (Alpha-Innotech, San Leandro, CA, U.S.A.).

NF-κB Luciferase Reporter Gene Assays

The Raw264.7 cells were cultured in a 24-well plate at a density of 2×10^4 cells/well and transfected on the following day. A dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A.) was used to determine the promoter activity. Briefly, the cells were transiently transfected with 1 μg/ml of the pNF-κB-luciferase plasmid and 20 ng of the pRL-SV plasmid (Promega, Madison, WI, U.S.A.) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, U.S.A.), and then exposed to LPS and/or *L. casei* 3260 for 16 h. The firefly and *Renilla* luciferase activities in the cell lysates were measured using a luminometer (Labsystems, Fluoroskan Ascent, FL, U.S.A.). The relative luciferase activity was calculated by normalizing the NF-κB promoter-driven firefly luciferase activity versus that of *Renilla* luciferase.

Statistical Analysis

A paired Student's *t*-test was used to assess any significant differences between the treatment groups. The criterion for statistical significance was set at $p<0.05$.

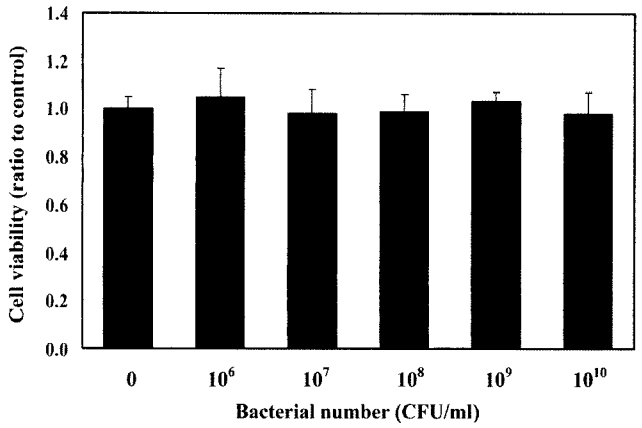

Fig. 1. Effect of *L. casei* 3260 on Raw 264.7 cell viability.
The cell viability was determined by a MTT assay after 24 h of incubation with the bacteria. Data shown are mean values with bars indicating the SD of the mean (n=3).

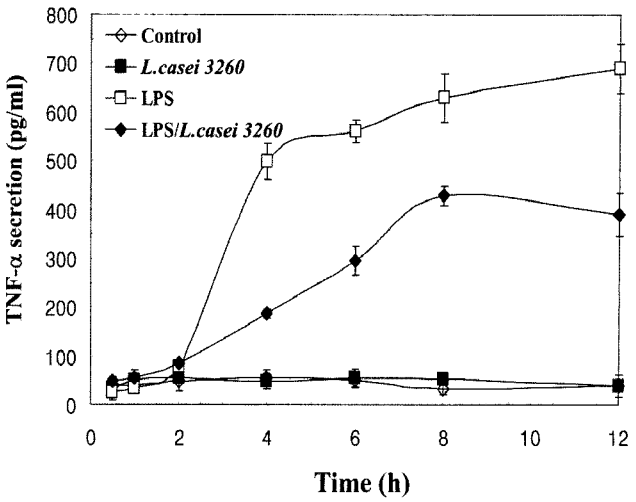

Fig. 2. Effect of *L. casei* 3260 on TNF-α secretion in LPS-stimulated Raw264.7 cells.
The level of TNF-α was determined by ELISA after 24 h of incubation with or without *L. casei* 3260. Data shown are mean values with bars indicating the SD of the mean from three separate experiments.

RESULTS AND DISCUSSION

Cell Viability and TNF-α Secretion

First, the cytotoxicity effect of *L. casei* 3260 on Raw264.7 cells was evaluated. The cells were treated with various numbers of *L. casei* 3260, ranging from 1×10^6 CFU/ml to 1×10^{10} CFU/ml, for 24 h and subjected to a MTT assay. As shown in Fig. 1, since treatment with *L. casei* 3260 did not exhibit cytotoxicity up to 1×10^{10} CFU/ml, all the subsequent experiments used 1×10^9 CFU/ml. Furthermore, the incubation of Raw264.7 cells with *L. casei* 3260 in the presence of 1 μg/ml LPS did not alter the cell numbers when compared with the control or cells treated with only LPS (data not shown). Therefore, these results confirmed that the *L. casei* 3260 data from all the subsequent experiments were not due to the reduction of cell viability. It has been reported that some *Lactobacillus* spp. selectively inhibit the proliferation of Vero cells at the level of 1×10^{10} CFU/ml [25]. However, in this study, *L. casei* 3260 did not affect the proliferation of Raw264.7 cells up to a bacterial number of 1×10^{10} CFU/ml, suggesting that lactic acid bacteria may affect cell proliferation in a bacterial species-dependent manner.

The effect of *L. casei* 3260 on TNF-α production was determined in Raw264.7 cells stimulated by 1 μg/ml of LPS. Whereas LPS significantly increased the TNF-α production from 2 h to 12 h (Fig. 2), the treatment of Raw264.7 cells with *L. casei* 3260 significantly inhibited the LPS-induced TNF-α production. As *L. casei* 3260 alone did not stimulate TNF-α production, there are two possible explanations for the inhibition of TNF-α production by *L. casei* 3260: (1) *L. casei* 3260 may bind to the surface molecules, thereby suppressing the signaling pathways for TNF-α production or (2) *L. casei* 3260 may bind to LPS, thereby inhibiting the interaction between LPS and the Raw264.7 cells. To investigate the latter case, Raw264.7 cells were stimulated by LPS 1 h prior to *L. casei* 3260 treatment, yet there was no significant difference in the TNF-α production (no data shown). Thus, it was concluded that *L. casei* 3260 may inhibit TNF-α production by suppressing the signaling pathways.

Effect of *L. casei* 3260 on PGE_2 Secretion and COX-2 Expression

The level of PGE_2 following COX-2 expression in macrophage or immune cells is increased as a cellular response during the inflammatory process induced by TNF-α or LPS. Therefore, the inhibitory effect of *L. casei* 3260 on PGE_2 production was investigated in LPS-stimulated Raw264.7 cells. The LPS treatment significantly increased PGE_2 production 14.2-fold when compared with the untreated control, whereas *L. casei* 3260 showed an adverse effect on PGE_2 production

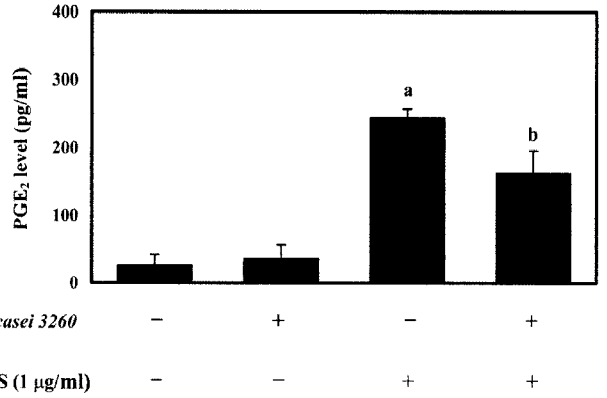

Fig. 3. Effect of *L. casei* 3260 on PGE_2 secretion in LPS-stimulated Raw264.7 cells.
The level of PGE_2 was determined by ELISA after 24 h of incubation with or without *L. casei* 3260. Data shown are mean values with bars indicating the SD of the mean from three separate experiments. a, $p<0.05$ compared with untreated control. b, $p<0.05$ compared with LPS-treated group.

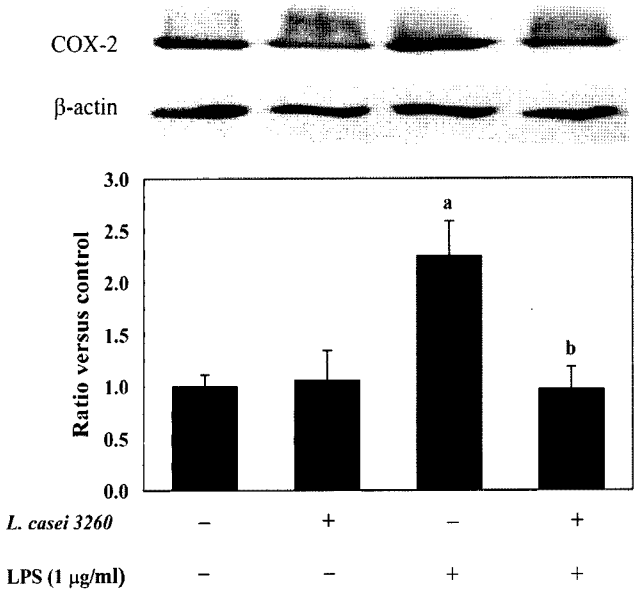

Fig. 4. Effect of *L. casei* 3260 on COX-2 enzyme expression in LPS-stimulated Raw264.7 cells.
The COX-2 level was assessed from 40 μg of a cytosolic protein extract prepared 24 h after exposing the cells to *L. casei* 3260. The total protein extract was applied to a 12% PAGE, and β-actin detected as the internal control. Data shown are mean values with bars indicating the SD of the mean from three separate experiments. a, $p<0.05$ compared with untreated control. b, $p<0.05$ compared with LPS-treated group.

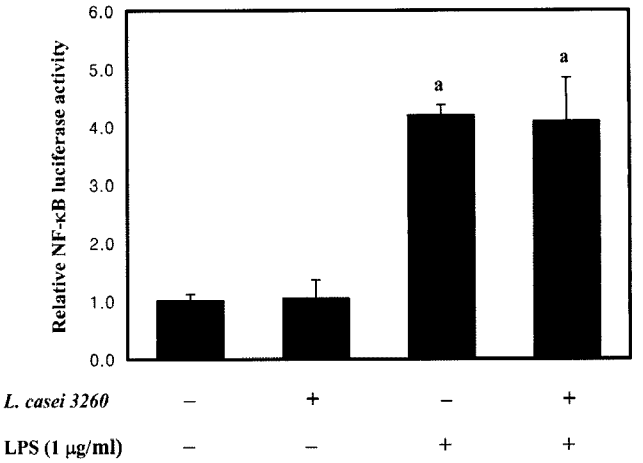

Fig. 5. Inhibitory effect of *L. casei* 3260 on transactivation of the NF-κB reporter gene.
The Raw264.7 cells were transfected with the pNF-κB-Luc plasmid and a reporter gene analysis was performed. Data shown are mean values with bars indicating the SD of the mean from three separate experiments. a, $p<0.05$ compared with untreated control.

(Fig. 3). Consequently, these data suggest that *L. casei* 3260 may be able to inhibit the inflammation mediated by macrophage cells.

It has been reported that LPS or TNF-α treatment causes the induction of COX-2 by upregulation of transcriptional factors such as NF-κB or the p38-mediated signaling pathway [2, 22]. Therefore, this study examined the effect of *L. casei* 3260 on LPS-induced COX-2 protein levels using a Western blot analysis. As expected, LPS significantly upregulated COX-2 expression, yet *L. casei* 3260 treatment alone did not affect COX-2 expression (Fig. 4), which is consistent with the results for the PGE_2 level, which was not elevated with bacterial treatment alone. Since the inhibitory effect of *L. casei* 3260 on COX-2 expression was also observed after LPS stimulation, a possible explanation may be the antioxidative ability of *L. casei* 3260. Other evidence would seem to suggest that oxidative stress or a cellular redox state may relate to COX-2 induction through NF-κB and/or other signaling pathways [12, 5]. According to a report by Lee *et al.* [14], *L. casei* 3260 exerts an antioxidative ability by lowering lipid peroxidation, while increasing the ability to chelate metal ions. Therefore, the antioxidative effect of *L. casei* 3260 may inhibit COX-2 induction by scavenging oxidative stress [14]. Conversely, *L. casei* 3260 treatment may influence NF-κB activation *via* translocation to the nucleus, thereby inducing COX-2 expression. Consequently, this study attempted to detect the inflammatory indexes, including NF-κB activation and I-κBα degradation.

Effect of *L. casei* 3260 on NF-κB Transcriptional Activity

To examine the involvement of the NF-κB transcriptional factor in regulating COX-2 gene expression, the NF-κB transcriptional activity was measured using a luciferase reporter gene assay. The pNF-κB-transfected control group revealed a very low level of NF-κB expression (Fig. 5). LPS treatment for 16 h caused an approximately 4.2-fold increase in luciferase reporter activity when compared with the pNF-κB-transfected control group, and treatment of the LPS-treated group with *L. casei* 3260 showed no significant difference in NF-κB transcriptional activity compared with that in the LPS-treated group. Consequently, these data would seem to indicate that the interaction of *L. casei* 3260 with the Raw264.7 cells did not directly regulate the NF-κB transcriptional activity. Thus, to determine the NF-κB activation, the effect of *L. casei* 3260 treatment on p65 release and subsequent I-κBα degradation was examined.

Effect of *L. casei* 3260 on p65 Release and I-κBα Degradation

LPS treatment significantly increased the cytosolic level of p65 and cytosolic I-κBα degradation in the Raw264.7 cells (Fig. 6). These increased levels of cytosolic p65 and I-κBα degradation were also reversed when the Raw264.7 cells were treated with *L. casei* 3260, suggesting that the inhibition of NF-κB activation is due to the prevention of I-κBα degradation and subsequent p65 nuclear translocation. Therefore, the present data imply that *L. casei* 3260 may have beneficial effects on macrophage-mediated inflammation. Since NF-κB can be activated by two major kinase-mediated upstream signaling pathways, mitogen-activated phosphokinase (MAPK) and I-κBα kinase (IKK) [13, 10, 24], treatment with *L. casei* 3260 may also act on these upstream kinases.

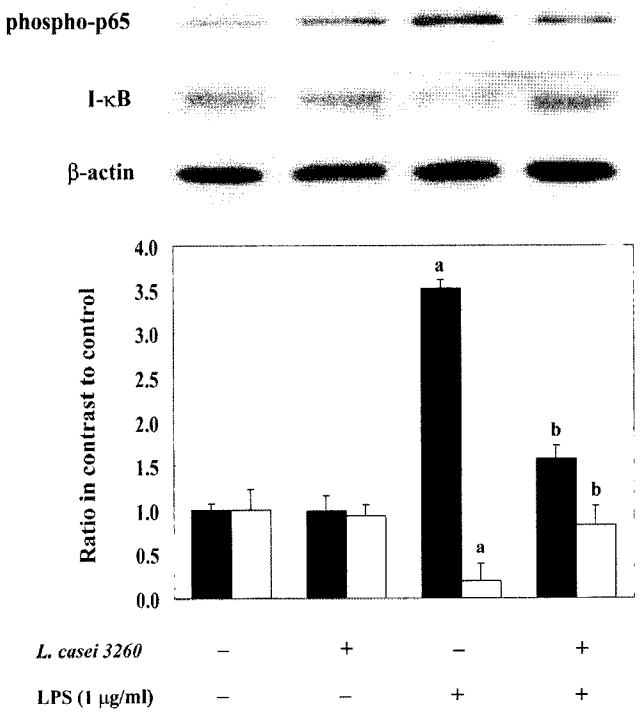

Fig. 6. Effect of *L. casei* 3260 on inhibition of p65 release and I-κB degradation.

The total cell extract was assayed for phospho-p65 and I-κB using a Western blot analysis. Forty μg of the total protein was applied to a 12% PAGE, and β-actin detected as the internal control. The closed bar indicates the relative ratio of phospho-p65 to β-actin, and the open bar indicates the relative ratio of I-κB to β-actin. Data shown are mean values with SD from three separate experiments. a, $p<0.05$ compared with untreated control. b, $p<0.05$ compared with LPS-treated group.

Thus, determining which kinases are inhibited by *L. casei* 3260 is a valuable topic for further study.

In conclusion, the present results indicate that *L. casei* 3260 exerts an antiinflammatory effect on LPS-stimulated Raw264.7 macrophage cells, at least in an *in vitro* system. This event would appear to be a result of NF-κB inactivation, as evidenced by the inhibition of p65 translocation and I-κBα degradation. Therefore, the consumption of *L. casei* 3260 may help in the inhibition of macrophage-mediated inflammation under pathological conditions.

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