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Conjugated linoleic acids produced by *Lactobacillus* dissociates IKK-γ and Hsp90 complex in *Helicobacter pylori*-infected gastric epithelial cells

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Although probiotics have been reported to reduce the gastric inflammatory response to *Helicobacter pylori* infection, little information is available regarding the molecular mechanisms behind this reduction. This study investigates the role of conjugated linoleic acids (CLA) produced by probiotics in interactions of IκB kinase (IKK) and heat shock protein 90 (Hsp90) to activate the nuclear factor-kappaB (NF-κB) signaling pathway in human gastric epithelial cells infected with *H. pylori*. Conditioned medium (CM) containing *Lactobacillus acidophilus*-producing CLA significantly inhibited the activated NF-κB signals and the upregulated expression of interleukin-8 (IL-8) in MKN-45 cells infected with *H. pylori*. Pretreatment with CM with CLA attenuated the increased IKK activity induced by *H. pylori*. Transfection of siRNA for IKK-β dramatically reduced *H. pylori*-induced IκBα phosphorylation, but siRNA for IKK-α had little effect on IκBα phosphorylation, although the siRNA for IKK-α significantly decreased IL-8 production. Furthermore, Hsp90 was associated with IKK-α and IKK-γ in *H. pylori*-infected cells, and CM with CLA dissociated the complex between Hsp90 and IKK-γ. These results suggest that CLA produced by probiotics has anti-inflammatory activity in gastric epithelial cells infected with *H. pylori* via dissociation of the IKK-γ and Hsp90 complex.

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Helicobacter pylori is a pathogen that has an important role in the pathogenesis of chronic gastritis, peptic ulcers, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma.[1,2] Persistent colonization of *H. pylori* in the human stomach results in the release of chemoattractants, such as interleukin-8 (IL-8). The released IL-8 induces the infiltration of neutrophils into the gastric mucosa, leading to chronic gastritis.

Nuclear factor-kappaB (NF-κB) is an important transcriptional factor that controls various biological processes, such as inflammation, cell survival or death, and the cell cycle. NF-κB dimers are stored in the cytoplasm in an inactive state by inhibitory proteins called IκBs. IκB kinase (IKK) is known to directly phosphorylate IκB, which then undergoes ubiquitin-mediated proteolysis, thereby releasing NF-κB dimers to translocate to the nucleus. The IKK complex contains three subunits: the catalytic subunits, IKK-α and IKK-β, and a regulatory subunit, IKK-γ (also known as NEMO, NF-κB essential modulator).[3–6] While IKK-α and IKK-β are essential for IκB phosphorylation, IKK-γ forms a tetrameric scaffold that can assemble two kinase dimers to facilitate trans-autophosphorylation.[7,8] Recently, heat shock protein 90 (Hsp90) has been found to associate stoichiometrically with the IKK complex, which may contribute to the stabilization, activation and/or shuttling of IKKs to the plasma membrane, since Hsp90 regulates the stability and function of a unique complement of signaling molecules.[9–12] Considering that the Hsp90–CDC37 chaperone complex has been implicated in the maturation of kinases and the IKK$\alpha/\beta/\gamma$ complex, and that the pharmacological inhibition of Hsp90–CDC37 by geldanamycin inhibits the TNF-α-mediated activation of NF-κB,[10,13] signal transduction to

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NF-κB activation by stimulators such as TNF-α includes Hsp90 molecules.

Several strains of bacteria that are considered to have probiotic effects, including *Lactobacilli* and *Bifidobacteria* species, are capable of converting linoleic acid to conjugated linoleic acid (CLA). CLA is a collective term used to describe a set of 28 distinct positional and geometric isomers of linoleic acid[14] and is most commonly found at positions *cis*-9, *trans*-11 (*c*9,*t*11) and *trans*-10, *cis*-12 (*t*10,*c*12), both of which possess biological activity.[15] CLA has been shown to exert numerous health benefits, including antiatherogenic, anti-diabetic, anti-inflammatory, and anticarcinogenic properties.[16] Although probiotics have been shown to reduce the gastric inflammatory response to *H. pylori* infection,[17] little information is available regarding the molecular mechanism for *Lactobacilli*-induced attenuation of gastric inflammation. This study asked whether CLA produced by *Lactobacillus acidophilus* may affect the NF-κB signal transduction pathway to upregulate the IL-8 inflammatory response to $CagA^+$ *H. pylori*. CLA produced by *L. acidophilus* is shown herein to reduce NF-κB activity and IL-8 expression through dissociation of the IKK and Hsp90 complex in human gastric epithelial cells infected with *H. pylori*.

MATERIALS AND METHODS

Cell Culture, *H. Pylori* Strain, and Assessment of CLA Produced by *L. Acidophilus*

The MKN-45 gastric epithelial cell line was maintained at 37°C in 5% CO_2 in RPMI-1640 medium, supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin).[18] $CagA^+$ *H. pylori* strain 60190 (ATCC 49503, *vacA* s1a/m1) was maintained under microaerophilic conditions in Brucella broth that was supplemented with 5% horse serum.[19] Bacteria were centrifuged at $3500 \times g$ for 5 min at 4°C and washed with phosphate-buffered saline (PBS). The bacterial concentrations were estimated using an optical density at 560 nm (OD560) of 0.1, as 4×10^7 CFU/ml *H. pylori*. MKN-45 cells in six-well plates were pretreated with conditioned medium (CM) containing CLA 1 h prior to the addition of *H. pylori* at a multiplicity of infection of 100. For the preparation of the CM, *L. acidophilus* (ATCC 832, approximately 1×10^7 CFU/100 ml of culture medium) was incubated for 18 h with 0.5 g/l of linoleic acid (Sigma Chemical Co., St Louis, MO, USA) in RPMI-1640, in which the bacteria were grown to the stationary phase. Live bacteria were removed by filtration through a 0.2 μm syringe filter, and the CM was placed onto the MKN-45 cells. Serial 100-fold dilutions of CM were plated on brain–heart infusion medium to ensure the absence of viable bacteria.

Amounts of CLA were measured as described previously.[20,21] *L. acidophilus* was incubated for 18 h at 37°C in 10 ml of RPMI-1640 or Mann–Rogosa–Sharpe broth in the presence or absence of 0.5 g/l of linoleic acid suspended in 0.05% Tween-80. Samples were centrifuged at $3500 \times g$ for 15 min, from which process supernatants were obtained. To extract lipids from the supernatants immediately, 24 ml of a 2:1 chloroform:methanol solution and 8 ml of 0.88% NaCl were mixed with 2 ml of medium. Ten milliliters of the lower layer of the mixture were dried under nitrogen at 40°C and resuspended in hexane. Fatty acid methyl esters were produced by incubating the samples with 40 μl methyl acetate and 80 μl sodium methoxide for 15 min at 50°C. Methylated fatty acids were subjected to gas chromatography on a Varian 3600 GC using a SP2560 column (Supelco). Integration and quantitation were performed using the Class-VP Chromatography Data System (version 4.2, Shimadzu Scientific Instruments). Purified *c*9,*t*11- and *t*10,*c*12-CLA isomers were obtained from Matreya (State College, PA, USA).

In some experiments, intestinal epithelial cells were pretreated with an NEMO-binding domain (NBD) peptide (200 μM, Peptron, Daejeon, Korea) for 1 h before addition of *H. pylori*. An NBD peptide can block the association of NEMO with the IKK complex and inhibit NF-κB activation.[22,23] Sequences of the wild-type and mutant peptides are drqikiwfqnrrmkwkkTALDWSWLQTE (wild) and drqikiwfqnrrmkwkkTALDASALQTE (mutant). Positions of the W→A mutations are underlined.[22]

Transfection and Reporter Assays

The reporter plasmids, pIL8-luciferase, p2x NF-κB-luciferase, and pβ-actin- and pRSV-β-galactosidase-luciferase transcriptional reporters, were provided by Dr Kagnoff of the University of California, San Diego.[24] In the reporter analysis, cells in six-well dishes were transfected with 1.5 μg of plasmid DNA using Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA, USA), as described previously.[25] The transfected cells were incubated for 48 h at 37°C in a 5% CO_2 incubator. Cells were then harvested, and whole cell lysates were prepared as described previously.[25] Luciferase activity was determined in accordance with the manufacturer's instruction (Tropix Inc., Bedford, MA, USA) and luminescence was quantitated for 10 s using a luminometer (MicroLumat Plus, Berthold GmbH & Co. KG, Bad Wildbad, Germany). Luciferase activity was determined and normalized relative to β-galactosidase expression in accordance with the manufacturer's instruction (Tropix Inc.). Briefly, β-galactosidase activity was determined using the chemiluminescent substrate AMPGD (3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricylo[3.3.1.1]decan]-4-yl)phenyl-β-D-galactopyranoside; Tropix Inc.) as described before.[18,20] Luminescence was induced by the addition of 50 μl 0.2 N NaOH containing 10% Emerald enhancer (Tropix Inc.) and quantitated for 10 s in a luminometer. Increased activity from pIL-8, p2x NF-κB, and pβ-actin promoters was calculated by comparing ratios of luciferase to β-galactosidase activities in the cells co-transfected with pIL8-luciferase and pRSV-β-galactosidase, p2x NF-κB-luciferase and pRSV-β-galactosidase, or pβ-actin-luciferase and pRSV-β-galactosidase, respectively. Non-transfected cells were used as a background control.[18,20,26]

RNA oligonucleotides for silencing IKK-α (5′-GCA GGCU CUUUCAGGGACA-3′), IKK-β (5′-GUGAAGAGGUGGUGG UGAGC-3′), and the nonsilencing control (5′-UUCUCCGAA CGUGUCACGU-3′) with two thymidine residues (dTdT) at the 3′ end were synthesized together with their corresponding antisense RNAs and then annealed (QIAGEN, Hilden, Germany), as described previously.[27] For knockdown of human hsp90 mRNA, siRNA for Hsp90 purchased the predesigned siRNA (siGENOME SMARTpool reagent) from Dharmacon (Lafayette, CO, USA).

Quantitative RT-PCR, Real-Time PCR, and Enzyme-Linked Immunosorbent Assay

Total cellular RNA from MKN-45 cells was extracted from the cells using an acid guanidinium thiocyanate–phenol–chloroform method (Trizol; GIBCO BRL, Gaithersburg, MD, USA). Quantitative RT-PCR for IL-8 and β-actin mRNA was performed using standard internal RNA, and real-time PCR was carried out using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Systems, Foster City, CA, USA) and SYBR green fluorescent dye, as described previously.[19] Probes, reagents, and TaqMan cytokine gene expression plates were used as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). IL-8 in culture supernatants was assayed by enzyme-linked immunosorbent assay (ELISA). Prior to measuring the IL-8 protein, the supernatants were filtered through a 0.22-μm filter to remove any contaminants. Human IL-8 was quantitated using a Quantikine immunoassay kit (R&D Systems, Minneapolis, MN, USA). To measure levels of phospho-IκBα, an IκBα ELISA kit was used (Active Motif, Carlsbad, CA, USA).

Electrophoretic Mobility Shift Assay

Cells were harvested and nuclear extracts were prepared as described previously.[18] Concentrations of protein in the extracts were determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Electrophoretic mobility shift assays (EMSA) were performed according to the manufacturer's instructions (Promega, Madison, WI, USA). In brief, 5 μg of nuclear extracts were incubated for 30 min at room temperature with a γ^{32}P-labeled oligonucleotide probe corresponding to a consensus NF-κB-binding site. After incubation, both bound and free DNAs were resolved on 5% native polyacrylamide gels, as described previously.[18] Supershift assays were used to identify the specific members of the NF-κB family activated by *H. pylori* infection. EMSA was performed as described above, except that rabbit antibodies (1 μg/reaction) against NF-κB proteins p50, p52, p65, c-Rel, and Rel B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added during the binding reaction period.[28]

Immunoblots

Cells were washed with ice-cold PBS and lysed in a 0.5 ml/well lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, 0.1% Triton X-100, 1 mM PMSF, and 10 μg/ml aprotinin). Fifteen to fifty micrograms protein per lane was size-fractionated on a 6% polyacrylamide minigel (Mini-PROTEIN II; Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane (0.1-μm pore size). Specific proteins were detected using mouse anti-human IκBα (Santa Cruz Biotechnology), IKK-α, IKK-β, IKK-γ, Hsp90, and actin (Cell Signaling Technology, Beverly, MA, USA) as primary antibodies, and peroxidase-conjugated anti-mouse IgG (Transduction Laboratories, Lexington, KY, USA) as a secondary antibody. Specifically bound peroxidase was detected by enhanced chemiluminescence (ECL system; Amersham Life Sciences, Buckinghamshire, England) and exposure to X-ray film.

Immunoprecipitation

For the immunoprecipitation assay, the cells were collected in lysis buffer (50 mM HEPES at pH 7.6, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 0.5 mM phenylmethyl sulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) after treatments as described in the figure legends. The lysates were mixed and precipitated with the relevant antibody and protein G-sepharose beads by incubation at 4°C for overnight. Anti-HA and anti-flag antibodies were purchased from Santa Cruz Biotechnology and Sigma, respectively. HA-tagged IKK-α, IKK-β, and IKK-γ constructs were kindly supplied by Dr Gang Min Hur at Chungnam National University, Korea.[29] For the detection of the modified IKK-γ protein, MKN-45 cells were lysed in a lysis buffer (20 mM Tris at pH 7.6, 0.5% NP-40, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, and 10 mM *N*-ethylmaleimide) and lysates were incubated with anti-IKK-γ antibody and protein G-sepharose. All immunoprecipitates were washed four times with lysis buffer, boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and resolved on an 8% polyacrylamide gel.[30]

In Vitro Kinase Assay

IKK activity on IκBα phosphorylation was determined by using an immunocomplex kinase assay, as described previously.[31] Cells were lysed in Triton lysis buffer containing protease and phosphatase inhibitors and then cleared by centrifugation at 14 000 r.p.m. for 10 min. Three hundred micrograms of whole cell extract was immunoprecipitated with anti-IKK-γ/protein-A beads, and the kinase reaction was performed by incubating 25 ml of kinase buffer containing 20 mmol/l of HEPES (pH 7.7), 10 mmol/l of $MgCl_2$, 5 mmol/l of dithiothreitol, 50 mmol/l of ATP, and 5 mCi of [γ-^{32}P] ATP with GST-IκBα substrate (Upstate Biotechnology Inc., Lake Placid, NY, USA) for 30 min at 30°C. The substrate protein was resolved by gel electrophoresis, and phosphate incorporation was assessed by autoradiography. HTScan IKK-β kinase assay kit was obtained from Cell Signaling Technology (Danvers, MA, USA). This contains GST-IKK-β kinase

protein, a biotinylated peptide substrate and a phosphoserine antibody for detection of the phosphorylated form of the substrate peptide. Assay was performed according to the manufacturer's instruction.

Statistical Analyses

Wilcoxon's rank sum test was used for the statistical analyses. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

L. acidophilus Produces CLA in Broth Culture Supernatants

Gas chromatography was used to measure the amounts of *c*9,*t*11- and *t*10,*c*12-CLA produced by *L. acidophilus*. The amounts of *c*9,*t*11- and *t*10,*c*12-CLA produced by *L. acidophilus* were 927.6 ± 51.8 μg/mg protein and 736.2 ± 32.6 μg/mg protein, respectively (mean ± s.e.m., $n = 5$).

Culture Supernatants Containing CLA Produced by *L. Acidophilus* Inhibit IL-8 Expression and NF-κB Activation in Gastric Epithelial Cells Infected with *H. Pylori*

Infection of MKN-45 cells with *H. pylori* for 24 h secreted IL-8 in higher amounts than that with the uninfected control. In this system, pretreatment with CM containing *L. acidophilus*-producing CLA significantly decreased the IL-8 releases induced by *H. pylori* in a dose-dependent manner (Figure 1).

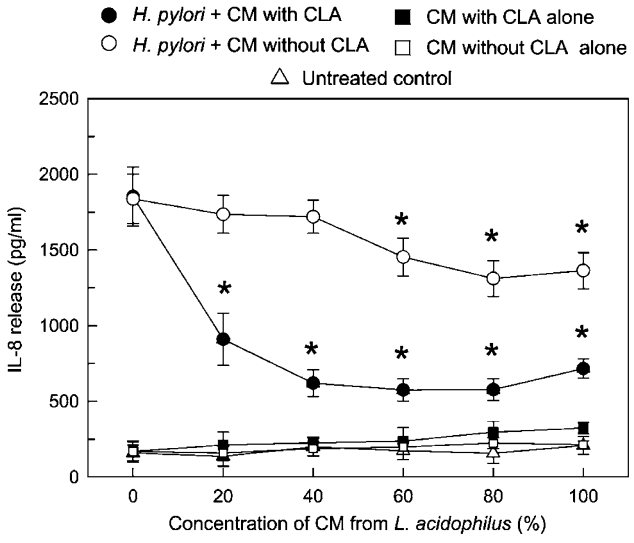

Figure 1 Effects of CM containing CLA on IL-8 release from MKN-45 gastric epithelial cells infected with *H. pylori*. MKN-45 cells were pretreated with the indicated concentration of CM with or without CLA for 1 h and were then combined with *H. pylori* for 24 h. The protein levels of IL-8 in the culture supernatants were determined by ELISA. Data are expressed as the mean ± s.e.m. ($n = 5$). Asterisks indicate values that are significantly different from each *H. pylori*-infected cell without CM ($P < 0.05$), as determined by Wilcoxon's rank sum test. ●, *H. pylori* + CM with CLA; ○, *H. pylori* + CM without CLA; ■, CM with CLA alone; □ CM without CLA alone; △, untreated control.

However, CM (0–40%) obtained from the incubation with *L. acidophilus* in the absence of linoleic acid did not show significant changes of the IL-8 releases induced by *H. pylori*. In this experiment, significant inhibitions of IL-8 release by CM without CLA were found in more than 60% of treated concentration, but their inhibitions were lower compared with those by CM from *L. acidophilus* with CLA.

To confirm the decrease of IL-8 release by CM containing *L. acidophilus*-producing CLA, the numbers of *IL-8* mRNA transcripts were measured by real-time PCR and quantitative RT-PCR using an internal RNA standard. The result of this experiment showed that the upregulated expression of *IL-8* mRNA was significantly reduced by the addition of CLA-containing CM (Figure 2a). However, the addition of CM

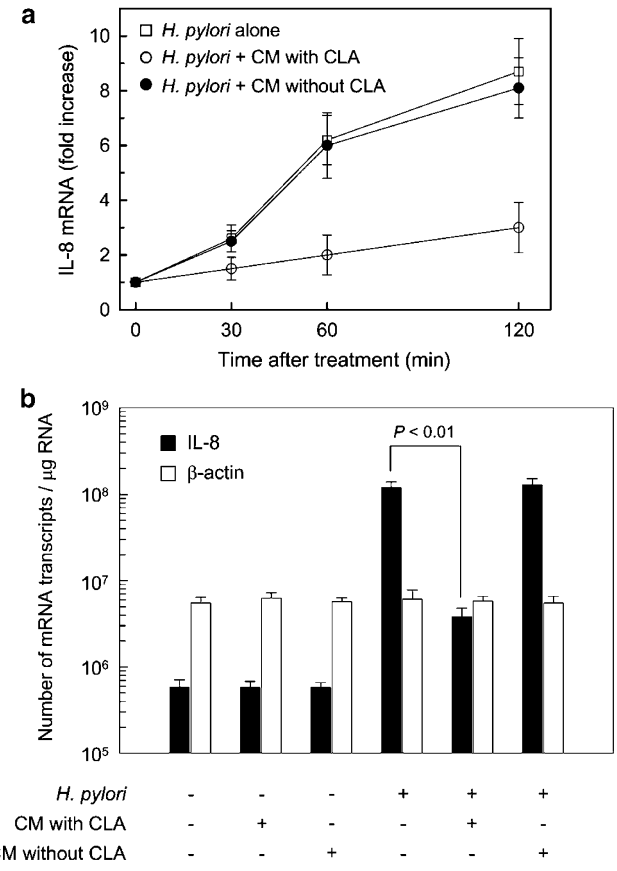

Figure 2 Effects of CM containing CLA on *IL-8* mRNA expression in MKN-45 gastric epithelial cells infected with *H. pylori*. (**a**) MKN-45 cells were treated with CM containing CLA (20%) and *H. pylori* for the indicated times. *IL-8* mRNA levels were detected by real-time PCR. Data are expressed as fold-increases in *IL-8* mRNA transcript levels relative to the uninfected control (mean ± s.e.m., $n = 5$). *β-actin* mRNA levels in each group remained relatively constant. □, *H. pylori* alone; ○, *H. pylori* + CM with CLA; ●, *H. pylori* + CM without CLA. (**b**) MKN-45 cells were pretreated with CM (20%) obtained from *L. acidophilus* for 1 h and were then combined with *H. pylori* for another 8 h. Total RNA was reverse-transcribed using an oligo(dT) primer and a synthetic internal *IL-8* or *β-actin* RNA standard and amplified by PCR. Data represent mean ± s.d. of the results from five different cultures. Statistical analysis was determined by Wilcoxon's rank sum test. ■, IL-8; □, *β*-actin.

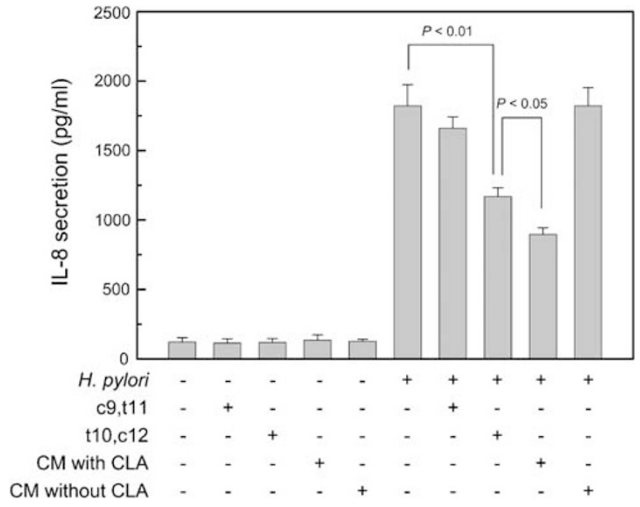

Figure 3 Effects of pure CLA isomers and CM from *L. acidophilus* on IL-8 secretion in *H. pylori*-infected MKN-45 cells. MKN-45 cells were pretreated with each pure CLA isomer (20 μM) or each indicated CM (20%) for 1 h and were then combined with *H. pylori* for 24 h. Protein levels of IL-8 in the culture supernatants were determined by ELISA. Data are expressed as the mean ± s.e.m. ($n = 5$). Statistical analysis was determined by Wilcoxon's rank sum test.

obtained from the incubation with *L. acidophilus* in the absence of linoleic acid did not show significant changes of the *IL-8* mRNA expression. In addition, as shown in Figure 2b, pretreatment with CM (20%) containing *L. acidophilus*-producing CLA significantly decreased *IL-8* mRNA expression induced by *H. pylori*, which is consistent with the results in Figure 1. In this experiment, the level of *β-actin* mRNA in each group remained relatively constant ($\sim 5 \times 10^6$ mRNA transcripts/μg RNA).

Since *c*9,*t*11 and *t*10,*c*12 are known to be the most commonly found among total CLA,[15] we performed an experiment to confirm whether pure *c*9,*t*11- and *t*10,*c*12-CLA isomers could downregulate IL-8 expression in *H. pylori*-infected gastric epithelial cells. Pretreatment with *t*10,*c*12-CLA significantly attenuated IL-8 secretion in MKN-45 cells infected with *H. pylori* ($P < 0.01$). However, *c*9,*t*11-CLA had little decrease of IL-8 secretion ($P = 0.082$) (Figure 3). Furthermore, inhibition by pretreatment with CM obtained from the incubation with *L. acidophilus* in the presence of linoleic acid was higher than that by pretreatment with pure *c*9,*t*11- and *t*10,*c*12-CLA isomers ($P < 0.05$). The IL-8 secretion by pretreatment with a mixture of *c*9,*t*11- and *t*10,*c*12-CLA isomers (20 μM each) did not show a synergistic effect (data not shown). Pretreatment with *t*10,*c*12-CLA (20 μM) also downregulated the increased activities of NF-κB and IKK in MKN-45 cells infected with *H. pylori*, measured reporter gene assay (*H. pylori*, 3.2 ± 0.4; *H. pylori* + *t*10,*c*12, 1.9 ± 0.3; *H. pylori* + *c*9,*t*11, 2.9 ± 0.3; mean fold induction ± s.e.m. of NF-κB luciferase activity relative to untreated control, $n = 3$; *H. pylori*, 3.9 ± 0.3; *H. pylori* + *t*10,*c*12, 2.3 ± 0.3; *H. pylori* + *c*9,*t*11, 3.5 ± 0.4; mean fold induction ± s.e.m. of IKK-β kinase activity relative to untreated controls, $n = 3$).

The transcription factor NF-κB is one component of a signaling pathway, which regulates IL-8 expression induced by *H. pylori* infection.[32] Consistent with this, infection with *H. pylori* increased NF-κB DNA binding, as shown by EMSA (Figure 4a), in which increased level of NF-κB was first detected 10 min after infection and increased continuously until the end of the experiment (1 h post-infection). Concurrently, degradation of IκBα was observed in *H. pylori*-infected cells. It was, therefore, asked whether CLA could prevent *H. pylori*-induced NF-κB transcriptional activity in MKN-45 cells. As shown in Figure 4b, *H. pylori* activated NF-κB signals and the addition of CM containing CLA suppressed the NF-κB activation in MKN-45 cells. Concurrently, *H. pylori* induced IκBα degradation in MKN-45 cells, which was significantly prevented in CM-pretreated cells. However, pretreatment with CM without CLA did not change the NF-κB activation in MKN-45 cells infected with *H. pylori* (Figure 4c). A supershift assay was performed to identify the specific NF-κB subunits that comprise the NF-κB signal detected by EMSAs in *H. pylori*-infected cells. Specific antibodies to p50, p52, p65, c-Rel, and Rel B were used for these experiments. Supershift studies demonstrated that antibodies to p65 and p50 shifted the NF-κB signals significantly. In contrast, anti-p52, anti-c-Rel or anti-Rel B antibodies did not shift the NF-κB signal (Figure 4d). These results suggest that NF-κB activation by *H. pylori* infection is mediated predominantly by heterodimers of p65/p50.

To confirm the inhibition of NF-κB activation and IL-8 expression by CM containing CLA, luciferase assays for NF-κB and IL-8 reporter genes were performed. Figure 5 shows that the activation of IL-8 and NF-κB transcriptional reporters was significantly inhibited in *H. pylori*-infected MKN-45 cells when pretreated with CM containing CLA. However, CM obtained from the incubation with *L. acidophilus* in the absence of linoleic acid did not show significant inhibition of the enhanced activities of IL-8 and NF-κB transcriptional reporters. These results indicate that the pretreatment with CM containing *L. acidophilus*-producing CLA significantly inhibits gastric epithelial inflammatory signals, such as NF-κB activation and IL-8 expression, induced by *H. pylori* infection.

H. Pylori Induces IKK Activation in MKN-45 Gastric Epithelial Cells, and Culture Supernatants Containing CLA Produced by *L. Acidophilus* Inhibit the Activated IKK Signals

Major pathways for NF-κB activation involve the activation of IKK, which is followed by IκB degradation.[3] Infection of MKN45 cells with *H. pylori* increased signals of phosphorylated IKK-α/β, which were first activated within 10 min of infection and reached their maximum after 60–120 min (Figure 6a). We next asked whether activated IKK signals might be associated with the NF-κB activation and IL-8

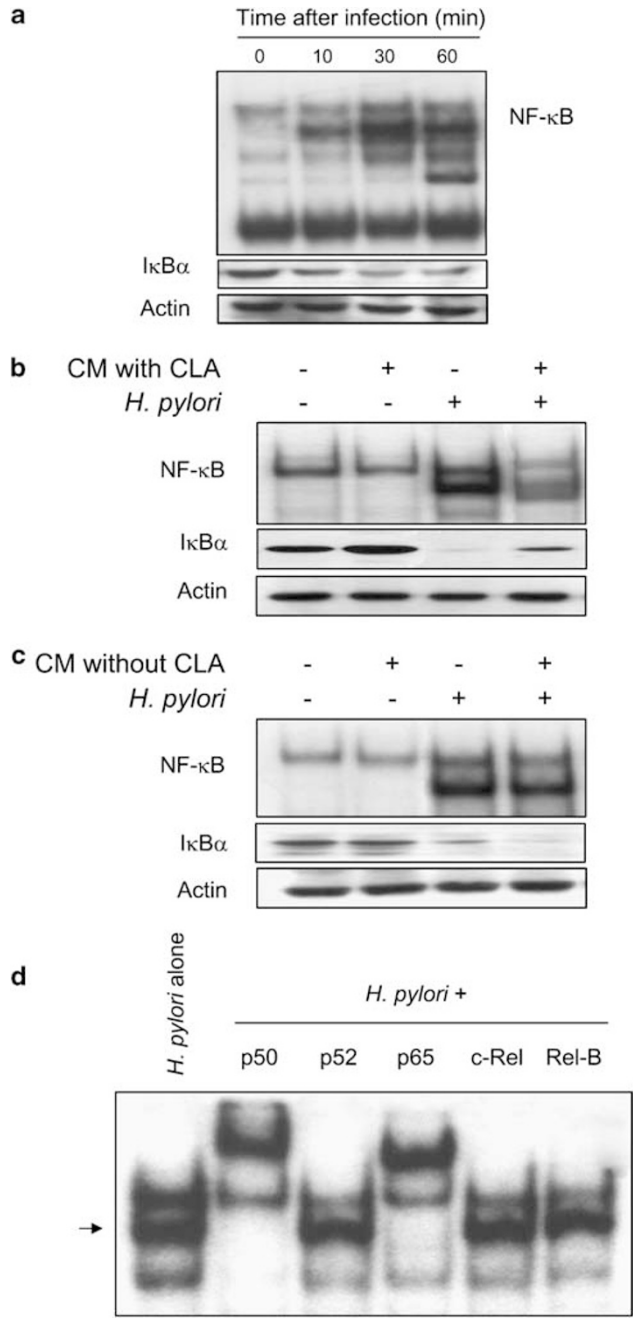

Figure 4 NF-κB activation and IκB degradation in MKN-45 gastric epithelial cells infected with *H. pylori*. (**a**) MKN-45 cells were infected with *H. pylori* for the indicated period. NF-κB DNA-binding activity was assessed by EMSA. Immunoblots for concurrent IκBα and actin levels in cells under the same conditions are provided beneath each EMSA. The results are representative of more than three repeated experiments. (**b** and **c**) MKN-45 cells were pretreated with CM containing CLA (B, 20%) or CM without CLA (**c**, 20%) for 1 h. CM-treated cells were then combined with *H. pylori* for another 2 h. NF-κB DNA-binding activity was assessed by EMSA. Immunoblots for concurrent IκBα and actin levels in cells under the same conditions are provided beneath each EMSA. The results are representative of more than five repeated experiments. (**d**) Supershift assays using nuclear extracts from MKN-45 cells infected with *H. pylori* for 2 h were performed using antibodies to p50, p52, p65, c-Rel, and Rel B. The results are representative of three repeated experiments.

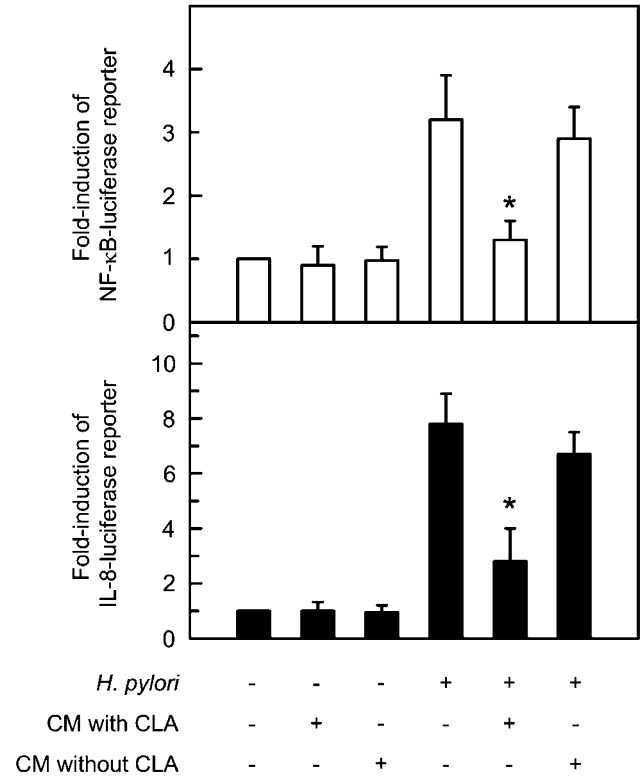

Figure 5 Effects of CM containing CLA on reporter gene activation in MKN-45 gastric epithelial cells infected with *H. pylori*. MKN-45 cells were transfected with pIL8- or p2x NF-κB-luciferase transcriptional reporter as indicated. After 48 h, the transfected cells were pretreated with CLA-contained CM (20%) or CLA-non-contained CM (20%) for 1 h and were then combined with *H. pylori* for 2 h (NF-κB) or 8 h (IL-8). Data are expressed as mean fold induction ± s.e.m. of luciferase activity relative to unstimulated controls ($n = 7$). The mean fold induction of the β-actin reporter gene activity relative to the unstimulated controls remained relatively constant throughout each experiment. $^*P < 0.05$ compared to *H. pylori* alone, as determined by Wilcoxon's rank sum test.

expression in *H. pylori*-infected epithelial cells. As shown in Figure 6b, the addition of an IKK inhibitor, NBD peptide, into MKN-45 cells significantly attenuated the increased NF-κB activation and IL-8 expression induced by *H. pylori*. To confirm the NBD peptide-induced IL-8 inhibition, an experiment using quantitative RT-PCR was performed. Similar findings were found with this experiment (control, $(7.2 \pm 1.1) \times 10^5$; NBD alone, $(6.8 \pm 1.4) \times 10^5$; mutant NBD alone, $(8.2 \pm 2.0) \times 10^5$; *H. pylori*, $(2.8 \pm 0.9) \times 10^7$; *H. pylori* + NBD, $(1.2 \pm 0.8) \times 10^6$; *H. pylori* + mutant NBD, $(2.3 \pm 0.8) \times 10^7$; mean number of mRNA transcripts ± s.d. per μg RNA in 8 h after infection, $n = 5$). In this experiment, the level of β-actin mRNA in each group showed relatively constant ($\sim 5 \times 10^6$ mRNA transcripts/μg RNA).

Since IKK-α and IKK-β are reported to be essential for IκBα phosphorylation and NF-κB activation,[4] it was necessary to determine which subunit of IKK is involved in IκBα phosphorylation when MKN-45 gastric epithelial cells were infected with *H. pylori*. As shown in Figure 7a, siRNA for

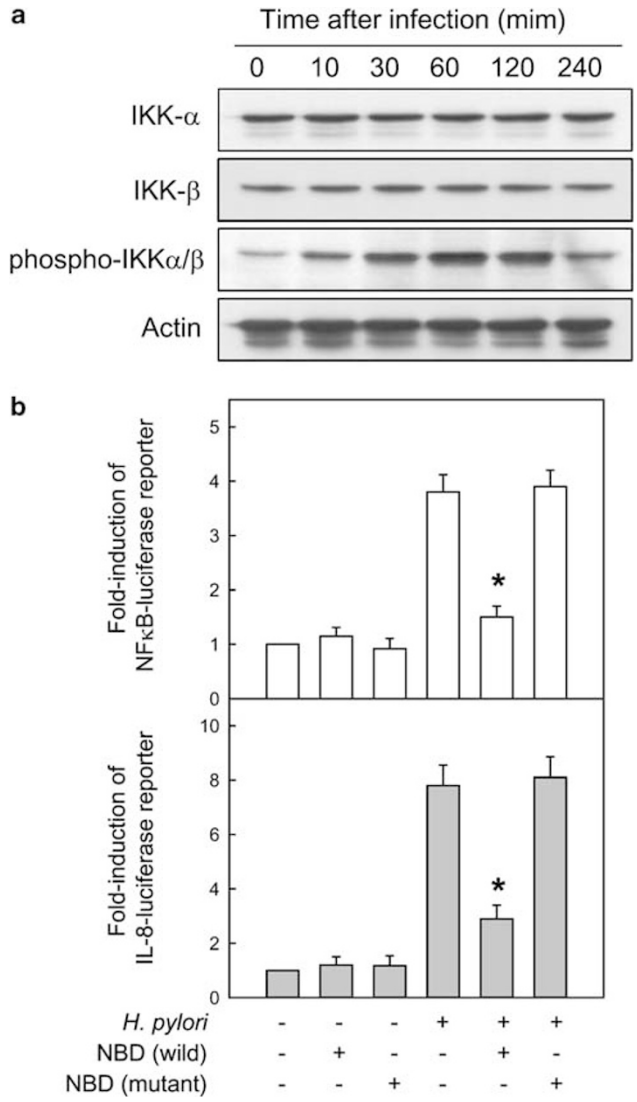

Figure 6 Suppression of IKK activity by CLA in MKN-45 gastric epithelial cells infected with *H. pylori*. (**a**) *H. pylori* induces IKK activation in MKN-45 gastric epithelial cells. MKN-45 cells were infected with *H. pylori* for the indicated period. Phosphorylation and protein expression of IKK-α, IKK-β, and actin were assessed by immunoblot. The results are representative of three repeated experiments. (**b**) Effects of IKK inhibition on NF-κB and IL-8 reporters in *H. pylori*-infected MKN-45 cells. MKN-45 cells were transfected with pIL8- or p2x NF-κB-luciferase transcriptional reporter as indicated. After 48 h, the transfected cells were preincubated with an NBD peptide (200 μM) for 1 h, *H. pylori* was then added for another 2 h (NF-κB) or 8 h (IL-8). Data are expressed as mean fold induction ± s.e.m. of luciferase activity relative to untreated controls ($n = 7$). The mean fold induction of β-actin reporter gene activity relative to untreated controls remained relatively constant throughout each experiment. Asterisks indicate values of *H. pylori* + NBD peptide that are significantly different from those of *H. pylori* alone ($P < 0.05$), as determined by Wilcoxon's rank sum test.

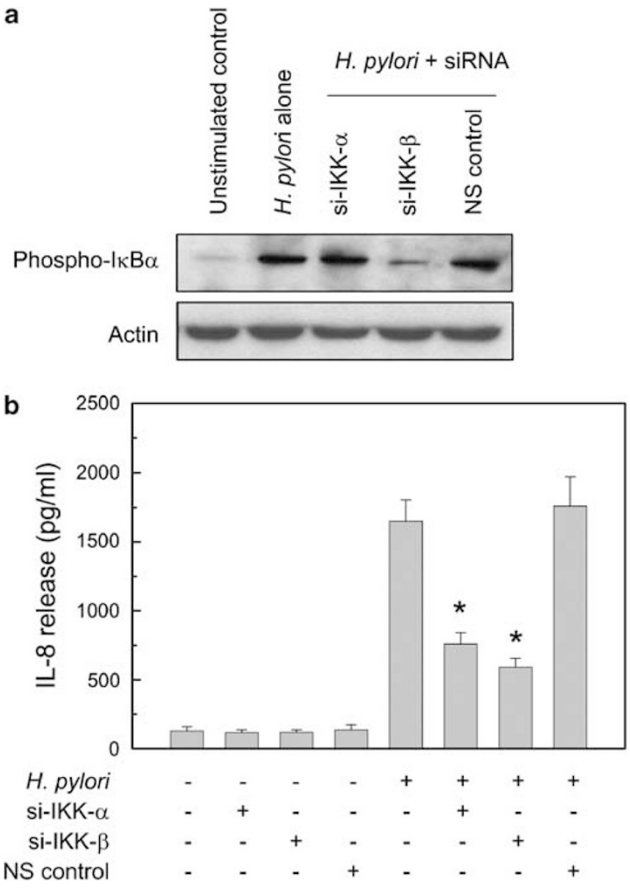

Figure 7 IKK is involved in the phosphorylation of IκBα induced by *H. pylori*. MKN-45 cells were introduced with siRNA for the control or each IKK for 48 h. (**a**) The siRNA-transfected cells were combined with *H. pylori* for 1 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies. Results shown are representative of three independent experiments. (**b**) The protein levels of IL-8 in culture supernatants obtained from 24 h after adding *H. pylori* were determined by ELISA. Data are expressed as mean ± s.e.m. ($n = 5$). Asterisks indicate values that are significantly different from *H. pylori*-infected cells without transfection ($P < 0.05$), as determined by Wilcoxon's rank sum test.

IKK-β dramatically reduced *H. pylori*-induced IκBα phosphorylation. In contrast, siRNA for IKK-α had little effect on IκBα phosphorylation, although the siRNA for IKK-α significantly decreased IL-8 production induced by *H. pylori* infection (Figure 7b).

To determine the effects of CM on IKK activity, an *in vitro* kinase assay for IKK was performed. As shown in Figure 8a, the infection of MKN-45 cells with *H. pylori* induced a strong increase of IKK activity, and pretreatment with CM containing CLA significantly reduced *H. pylori*-induced IKK activity. To quantify the inhibition of IKK activity, HTScan IKK-β kinase assay was performed. Pretreatment with CM containing CLA significantly prevented IKK-β activity by ~70% ($P < 0.01$), while pretreatment with CM obtained from the incubation with *L. acidophilus* in the absence of linoleic acid had no significant effect (Figure 8b). In this system, heat-killed *H. pylori* slightly increased IL-8 production in MKN-45 gastric epithelial cells (control, 127.7 ± 33.2; heat-killed *H. pylori*, 343.3 ± 52.9; live *H. pylori*, 1794.0 ± 174.4; $n = 3$, mean ± s.e.m.). However, an experiment for IKK activation stimulated with heat-killed *H. pylori* showed that

heat-killed *H. pylori* could not significantly activate IKK-β in MKN-45 gastric epithelial cells (live *H. pylori*, 3.3 ± 0.5; heat-killed *H. pylori*, 1.1 ± 0.3; $n = 5$, mean fold induction for control ± s.e.m.).

To confirm the hypothesis of the Hsp90–IKK–NF-κB mechanism, levels of phospho-IκBα were measured. Since detection of phospho-IκBα is difficult because of the short half-life of phospho-IκBα, we used a commercial IκBα ELISA kit. The results showed that *H. pylori* increased the levels of phospho-IκBα in MKN-45 cells. In contrast, CLA-containing CM (20%) significantly reduced the levels of phospho-IκBα (Figure 8c).

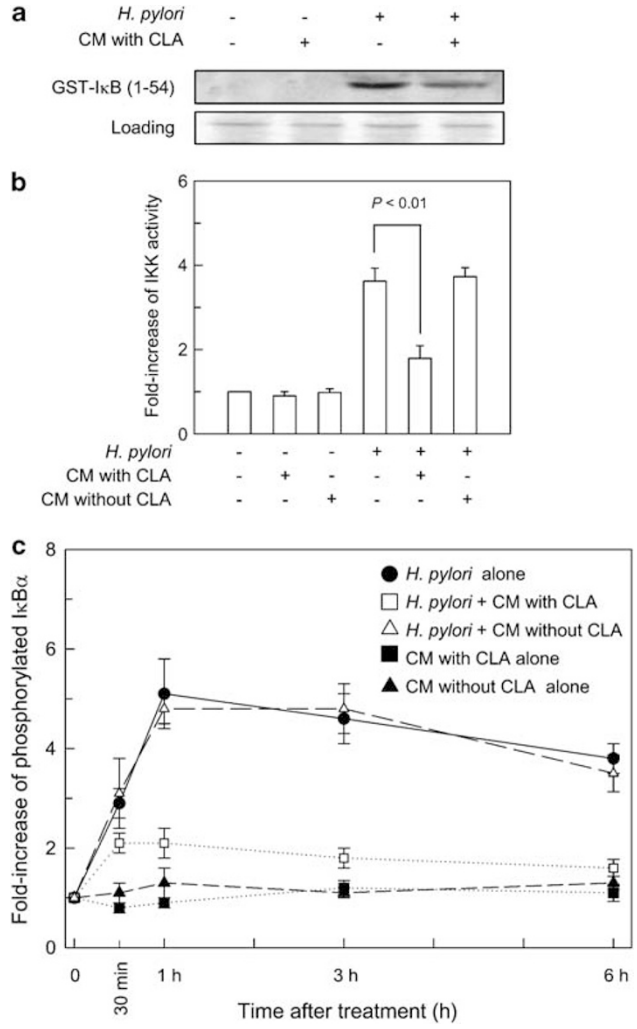

Figure 8 Suppression of IKK activity by CLA in MKN-45 gastric epithelial cells infected with *H. pylori*. (**a**) MKN-45 cells were pretreated with CM containing CLA (20%) for 1 h and were then combined with *H. pylori* for an additional 30 min. Whole cell extract was immunoprecipitated with anti-IKK-γ/protein-A beads, and kinase reactions were performed using glutathione-*S*-transferase-IκB as a substrate. Substrate protein was resolved by gel electrophoresis, and phosphate incorporation was assessed by autoradiography and by PhosphorImager analysis. Results shown are representative of three independent experiments. (**b**) IKK kinase activity was measured by HTScan IKK-β kinase assay kit. Data are expressed as mean fold induction ± s.e.m. of kinase activity relative to untreated controls ($n = 5$). Statistical analysis was determined by Wilcoxon's rank sum test. (**c**) Inhibition of IκBα phosphorylation in *H. pylori*-infected MKN-45 cells by CM containing CLA (20%). IκBα phosphorylation was measured by an IκBα ELISA kit. Data are expressed as mean fold induction ± s.e.m. of phosphorylated activity relative to untreated controls ($n = 3$).

Hsp90 is Associated With IKK Proteins in MKN-45 Gastric Epithelial Cells

H. pylori is known to induce the phosphorylation of Hsp90 in gastric epithelial cells.[33] In addition, Hsp90 is directly associated with the kinase domains of IKK-α and IKK-β, and they contribute to the stabilization, activation, and/or translocation of IKK.[9,10] On the basis of these results, it was asked whether Hsp90 might play a role in regulating the *H. pylori*-induced IKK activation in MKN-45 cells. First, the interactions of Hsp90 with IKK proteins in MKN-45 cells were examined when each protein of IKK-α, IKK-β, and IKK-γ was overexpressed in the cells. Interestingly, this experiment showed that IKK-β was unable to interact with Hsp90 (Figure 9), indicating that the interaction of IKK-β and Hsp90 is presumably indirect in MKN-45 cells. In contrast,

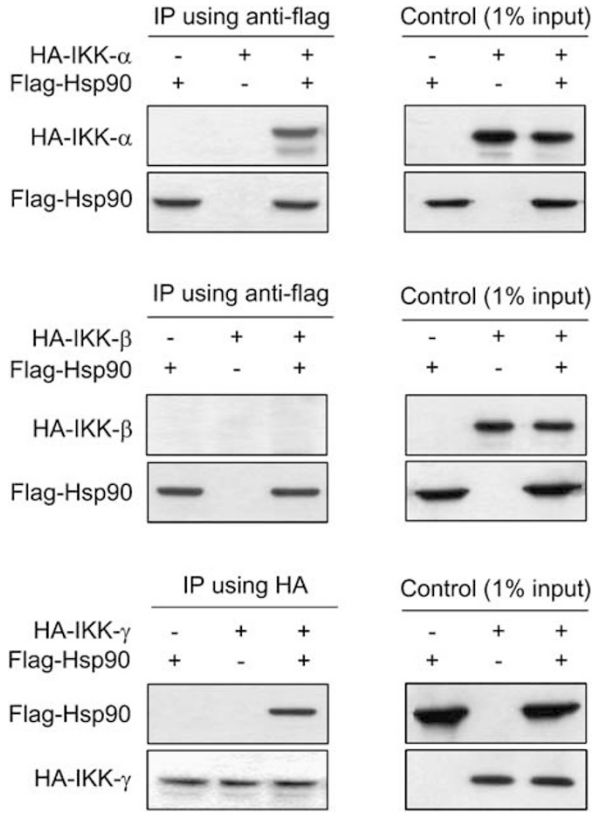

Figure 9 Hsp90 is associated with IKK complex in MKN-45 gastric epithelial cells. MKN-45 cells were transfected with HA-tagged IKK-α, IKK-β, and IKK-γ expression plasmids and flag-tagged Hsp90 expression plasmid. Immunoprecipitates containing Hsp90 and IKK-γ were prepared with anti-flag and anti-HA antibodies, and immunoprecipitants were analyzed by western blotting with anti-HA and anti-flag antibodies. Results shown are representative of at least three independent experiments.

IKK-α and IKK-γ strongly interacted with Hsp90 in each co-immunoprecipitation assay (Figure 9).

To determine whether Hsp90 is associated with expression of IL-8 gene, experiments of transfection with Hsp90 siRNA were performed. As shown in Figure 10a and b, transfection with siRNA for Hsp90 resulted in significant reduction of reporter gene activity of NF-κB and IL-8 in *H. pylori*-infected MKN-45 cells. In addition, a kinase assay for IKK showed that transfection with Hsp90 siRNA significantly prevented *H. pylori*-induced IKK activation, although infection of MKN-45 cells with *H. pylori* induced a significant increase in IKK activity (Figure 10c). These results suggest that the Hsp90–IKK complex plays an essential role in the activation of NF-κB and IL-8 gene in gastric epithelial cells in response to *H. pylori* infection.

Culture Supernatants Containing CLA Produced by *L. Acidophilus* Induces Dissociation of the Hsp90 and IKK-γ Complex in MKN-45 Cells Infected With *H. Pylori*

In the previous study, we have demonstrated that IKK-α and -γ strongly interacted with Hsp90 in MKN-45 gastric epithelial cells. To determine whether the interaction of IKK proteins with Hsp90 could be altered by treatment with CM containing CLA, *H. pylori*-infected MKN-45 cells treated with CM were lysed and immunoprecipitated with anti-IKK-γ and the subjected to immunoblot analysis for Hsp90 and IKK proteins. As shown in the top panel of Figure 11a, the interaction between IKK-γ and Hsp90 was reduced after 30 min of treatment with CM. In this experimental system, upregulated expression of IL-8 mRNA was significantly reduced by the addition of CLA-containing CM, using real-time PCR (Figure 2a). However, the addition of CM obtained from the incubation with *L. acidophilus* in the absence of linoleic acid did not show significant changes of the interaction between IKK-γ and Hsp90 (Figure 11b).

DISCUSSION

This report examined the impact of CLA produced by *L. acidophilus* on the Hsp90–IKK complex of *H. pylori*-infected gastric epithelial cells. CLA significantly decreases IKK activity by dissociation of the complex composed of IKK-γ and Hsp90. This phenomenon is then associated with both the suppression of IκB phosphorylation and NF-κB activation, which finally results in the inhibition of IL-8 expression in gastric epithelial cells infected with *H. pylori*.

Lactobacillus species are commensal in the human alimentary tract, and their concentrations in the normal stomach vary between 0 and 10^3/ml. As acid-resistant organisms, they persist in the stomach longer than other bacteria: dietary strains of bifidobacteria and *Lactobacilli* survive in high proportions (>80%) in the gastric environment for periods of 2 h.[34] Specific strains of *Lactobacillus* exert *in vitro* bactericidal effects against *H. pylori* through the release of bacteriocins or the production of organic acids and/or the inhibition of its adhesion to epithelial cells.[17] In addition,

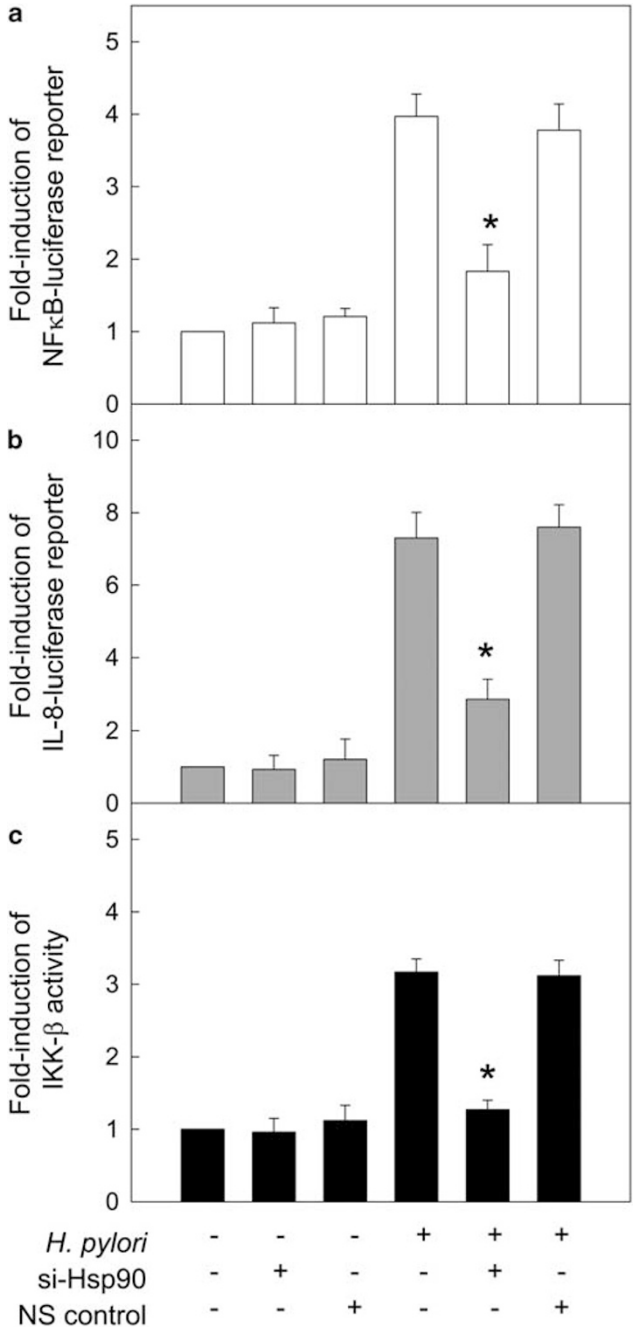

Figure 10 Transfection with siRNA for Hsp90 inhibits the activation of IKK and NF-κB and the expression of IL-8 in MKN-45 cells infected with *H. pylori*. (**a** and **b**) MKN-45 cells were transfected with siRNA for Hsp90 siRNA for 48 h. The siRNA-transfected cells were then co-transfected with pIL8- or p2x NF-κB-luciferase transcriptional reporter for another 24 h. *H. pylori* was added to co-transfected cells for 2 h (NF-κB, **a**) or 8 h (IL-8, **b**). Data are expressed as mean fold induction ± s.e.m. of luciferase activity relative to untreated controls ($n = 5$). The mean fold induction of β-actin reporter gene activity relative to untreated controls remained relatively constant throughout each experiment. (**c**) IKK kinase activity was measured by HTScan IKK-β kinase assay kit. Data are expressed as mean fold induction ± s.e.m. of kinase activity relative to untreated controls ($n = 5$). Asterisks indicate values of *H. pylori* + siRNA that are significantly different from those of *H. pylori* alone ($P < 0.05$), as determined by Wilcoxon's rank sum test.

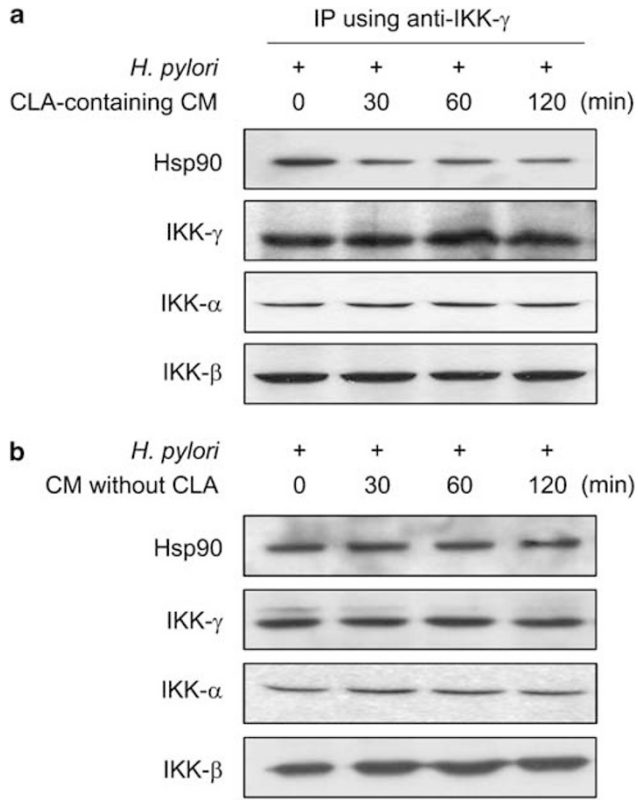

Figure 11 CLA produced by *L. acidophilus* alters the IKK-γ association with Hsp90 in MKN-45 cells infected with *H. pylori*. (**a**) MKN-45 cells were treated with CM containing CLA (20%) and *H. pylori* for the indicated times. Cell extracts from each sample were immunoprecipitated with anti-IKK-γ antibody. Immunoprecipitants were analyzed by western blotting with anti-Hsp90, anti-IKK-α, anti-IKK-β, and anti-IKK-γ antibodies. Western blotting using one percent of the cell extracts from each sample, as a control for protein input did not show significant changes in each sample. The results shown are representative of at least three independent experiments. (**b**) Culture conditions and experimental protocols were identical as in (**a**), except that CM without CLA (20%) was used. The results shown are representative of at least three independent experiments.

some strains of *Lactobacillus* are reported to reduce pro-inflammatory chemokine expression during *H. pylori* infection.[35,36] However, there exists little evidence regarding the molecular mechanism of *Lactobacilli*-induced suppression of inflammatory responses to *H. pylori* infection. Therefore, the findings of this study, including that CLA can suppress IL-8 expression and NF-κB activation in gastric epithelial cells infected with *H. pylori* through dissociation of the IKK-γ–Hsp90 complex, support a new mechanism of probiotic action against inflammatory response to infection with *H. pylori*.

Activated IKK induces IκB phosphorylation, which is followed by NF-κB activation.[3] An experiment described in this study showed that treatment with CM containing CLA blocked *H. pylori*-induced IKK activity and decreased IL-8 expression. These results suggest that CLA affects an IKK site in the signaling pathway of gastric inflammation induced by *H. pylori* infection. In the previous study, we have demonstrated that IKK-β may play a more important role in *H. pylori*-induced IκBα phosphorylation compared with IKK-α. In addition, IKK-α and IKK-γ strongly interacted with Hsp90 in MKN-45 gastric epithelial cells. Hsp90 is known to play an important role in signaling transduction networks and serves a critical function both in facilitating the biosynthesis of components of the IKK complex and in maintaining the mature forms of the kinase complex in a conformation that allows for its eventual biochemical function and stability.[9,10] Along this line of reasoning, inhibition of Hsp90 significantly downregulates the increased IKK activity and IL-8 expression in *H. pylori*-infected MKN-45 cells, suggesting an important role for Hsp90 in the signaling mechanism of inflammatory responses to *H. pylori* infection. Furthermore, Hsp90 seems to be directly associated with IKK-γ in gastric epithelial cells infected with *H. pylori*. Experiments from this study supported this hypothesis; the interaction between IKK-γ and Hsp90 was reduced after treatment with CM containing CLA. Concurrently, the interruption of the association between IKK-γ and Hsp90 by CLA resulted in the decrease of IL-8 mRNA expression. These results suggest that CLA produced by *L. acidophilus* may induce the dissociation of the complex between Hsp90 and IKK-γ molecules in MKN-45 gastric epithelial cells infected with *H. pylori*.

Hsp90 can regulate Nod1, which induces inflammatory responses to bacterial infection using the components of the bacterial cell well[37,38] and *H. pylori* also induces the phosphorylation of Hsp90 in gastric epithelial cells.[33] Therefore, it is possible that pretreatment with CLA-containing CM may affect the expression of Hsp90 molecules in *H. pylori*-infected gastric epithelial cells. Further study is needed to clarify this possibility.

H. pylori can activate MAPK signaling pathway to IL-8 production in gastric epithelial cells[39] and MAPK may be associated with NF-κB activation, suggesting an association between MAPK and IKK activation in *H. pylori*-infected gastric epithelial cells. Considering that CLA from *L. acidophilus* do not completely inhibit *H. pylori*-induced IL-8 production and NF-κB activation in the present study, there may be other pathways that sustain the ability of *H. pylori* to induce proinflammatory cytokine production, including MAPK and AP-1 pathway. In addition, there is a possibility that CLA may block translocation of NF-κB into the nucleus or that it may exert effects on ubiquitination in intestinal epithelial cells, indicating that further study is needed to clarify other contributing factors to account for the CLA-induced decrease of IL-8 expression in gastric epithelial cells infected with *H. pylori*.

Several clinical trials have shown that probiotics generally do not eradicate *H. pylori*, although they decrease the density of colonization, thereby maintaining lower levels of *H. pylori* in the stomach.[40,41] On the other hand, the antioxidant and anti-inflammatory properties exerted by probiotics may stabilize the gastric barrier function and decrease mucosal

inflammation.[17] As suggested by the 2000-Maastricht Consensus Conference on *H. pylori*, probiotic microorganisms may be used as a possible tool for the management of *H. pylori* infection and its associated gastric inflammation.[42] The findings of this study provide evidence that CLA produced by probiotics can attenuate gastric inflammatory responses associated with *H. pylori* infection.

There are numerous CLA isomers present in CM that may have been obtained from the incubation of *L. acidophilus* in the presence of linoleic acid. However, the present study measured only two isomers of CLA, *c*9,*t*11 and *t*10,*c*12, because these isomers are known to be most commonly found among total CLA.[15] It was asked whether pure isomers of CLA have inhibitory effects on IL-8 expression in *H. pylori*-infected MKN-45 cells. Pretreatment with pure *t*10,*c*12-CLA isomer significantly attenuated IL-8 secretion in MKN-45 cells infected with *H. pylori*. In contrast, *c*9,*t*11-CLA showed a tendency of decreasing IL-8 secretion but this inhibition had no significant value ($P = 0.082$), suggesting that different isomers of CLA exert differential effects on gastric epithelial cells infected with *H. pylori*. In addition, inhibition by pretreatment with CM containing *L. acidophilus*-producing CLA was significantly higher than that by pretreatment with pure *c*9,*t*11- and *t*10,*c*12-CLA isomers. These results also indicate that other components in CM obtained from *L. acidophilus* in the presence of linoleic acid may contribute to inhibiting inflammatory responses to *H. pylori* infection. Therefore, further study is needed to clarify the biochemical effects on *H. pylori* infection of CLA isomers or other components that may have been present in the CM.

In summary, this study has demonstrated that CLA produced by *L. acidophilus* has anti-inflammatory activity in gastric epithelial cells infected with *H. pylori* through the dissociation of IKK-γ and Hsp90 complex. This CLA action is novel in the inhibition of the inflammatory responses to *H. pylori* infection, suggesting that CLA produced by probiotics may play a role in the host defense system.

ACKNOWLEDGEMENT

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