Inhibitory effects of tilianin on the expression of inducible nitric oxide synthase in low density lipoprotein receptor deficiency mice

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Abstract

We investigated the effect of tilianin upon inducible nitric oxide synthesis in the plasma of low-density lipoprotein receptor knock-out (*Ldlr-/-*) mice fed with high cholesterol diet and in primary peritoneal macrophages of *Ldlr-/-* mice. High cholesterol diet induced nitric oxide production in the plasma of *Ldlr-/-* mice. Tilianin reduced the level of nitric oxide (NO) in plasma from *Ldlr-/-* mice induced by the high cholesterol diet. Tilianin also inhibited the NO production from the primary culture of peritoneal macrophages treated with lipopolysaccharide. The inhibition of NO production was caused by the suppression of inducible nitric oxide synthase (iNOS) gene expression in peritoneal macrophages isolated from *Ldlr-/-* mice. Moreover, tilianin inhibited the transcriptional activation of iNOS promoter that has NF-κB binding element. Thus, these results provide the first evidence that tilianin inhibit iNOS expression and production of NO and may act as a potential anti-inflammatory agent.

Keywords: anti-inflammatory agents; hyperlipoproteinemia; nitric oxide synthase type II; nitric oxide; tilianin

Introduction

NO is a gaseous free radical with very short half-life in biological system and can act both as a weak oxidant and as a reductant (Kanner *et al.*, 1991). NO is synthesized in mammals by the nitric oxide synthases (NOS). There are three NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Unlike eNOS and nNOS, iNOS is not constitutively expressed in macrophages and smooth muscle cells of inflammatory vascular tissues. In human atherosclerotic lesions, however, all three isoforms can be demonstrated (Wilcox *et al.*, 1997). Especially, iNOS expression has been localized to vascular smooth muscle cells and mononuclear leukocytes in early and advanced atherosclerosis (Esaki *et al.*, 1997).

Murine iNOS expression has been observed in various types of cells, including macrophages (Hibbs *et al.*, 1988), endothelial cells (Gross *et al.*, 1991), smooth muscle cells (Beasley *et al.*, 1991), and cardiac myocytes (Schulz *et al.*, 1992). In human patients with infectious or inflammatory disease, iNOS is most readily observed in monocytes or macrophages (MacMicking *et al.*, 1997). Therefore, in contrast to low level of NO produced by eNOS (Wever *et al.*, 1998), excessive NO prouction by iNOS in macrophages is considered to be proatherogenic (Buttery *et al.*, 1996; MacMicking *et al.*, 1997). NO interacts with superoxide to form the strong oxidant peroxynitrite (ONOO$^-$) (Beckman *et al.*, 1990), which induces lipid peroxidation and nitrosylation of tyrosine residue in human atherosclerotic lesions (Beckmann *et al.*, 1994; Wilcox *et al.*, 1997). In addition, it has been shown that suppression of NO by NOS inhibitor or iNOS gene deficiency (iNOS-/-) resulted in reduced atherosclerosis (Behr-Roussel *et al.*, 2000; Detmers *et al.*, 2000). Thus excessive NO has the potential to exacerbate

atherosclerosis.

In our previous study, we have reported that tilianin, a major component of *Agastache rugosa* (Labiatae) significantly inhibits the tumor necrotic factor-α (TNF-α)-induced expression of vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVEC) as its potential anti-atherogenic mechanism (Hong *et al*., 2001; Nam *et al*., 2005). However, it has not been determined the effect of tilianin on NO production. Here we report that tilianin potently inhibited NO production by iNOS, another possible anti-atherogenic mechanism. We have demonstrated the inhibitory effects in *in vivo* as well as *in vitro*.

Materials and Methods

Animal model

All animal experiments were approved by Institutional Animal Care and Use Committees (IACUC) of Ewha Womans University and followed National Research Council Guidelines. Twenty-five low density lipoprotein receptor-null (*Ldlr-/-)* male mice were divided into three groups (normal diet fed, *n* = 5, high-cholesterol diet (HCD) only; *n* = 10, HCD supplemented with tilianin; *n* = 10). The HCD contained 1.25% cholesterol, 6% fat and 0.5% cholic acid (CRF-1, Oriental Yeast Co., Ltd., Chiba, Japan). Tilianin was supplemented with 0.1% (wt/wt) of HCD. After 8 week experiment, plasma from the mice were collected and subjected to the measurement of total nitric oxide.

Histological finding and measurement of fatty streak lesion

The frozen sections of the aortic sinus were reacted with a rabbit anti-mouse iNOS antibody (Santa Cruz Biotechnology, CA) and a rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, NY) at 1:200 dilutions, then the reactivities were detected using ABC kit (Vector Laboratory, CA). Fatty streak lesions were quantified by evaluating the lesion size in the aortic sinus, as previously described (Paigen *et al*., 1987). All hearts were sectioned using a cryostat at -20°C and six consecutive 9 μm-thick sections were cut from the aorta where the valve cusp becomes visible. Atherosclerotic plaque was stained with Oil red O and counter-stained with Harris hematoxylin. The lesion area was then quantified by computer-assisted morphometry (Image pro plus, MD) and the average lesion size was calculated for each animal.

Determination of NO synthesis

Nitric oxide (NO) production was measured by monitoring levels of nitrite plus nitrate (NO$_x$) in plasma using automatic NO analyzer described previously (Morita *et al*., 1994). Nitrite in culture media was measured spectrophotometrically as described (Sherman *et al*., 1993) using the Griess reagent.

Culture of peritoneal macrophages

Mice were injected with 2 ml of 4% thioglycolate broth. After 6 days, peritoneal macrophages were isolated from peritoneal lavage of mice. Peritoneal macrophages were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Immunoblot analysis

The isolated peritoneal macrophages were incubated with tilianin (1, 10, 100 μM) for 2 h and immediately activated by LPS (2 μg/ml) at 37°C for 18 h. The macrophages were harvested, washed with PBS containing 0.1 mM PMSF, and lysed by three cycles of freezing and thawing in liquid nitrogen. Cytosolic fraction was obtained by centrifugation at 12,000 *g* at 4°C for 10 min. The concentration of protein was analyzed by the Lowery method. Samples (50 μg) were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NY). The detection of iNOS was performed with anti-iNOS antibody (Santa Cruz Biotechnology, CA) and ECL western blotting analysis system (Amersham, UK).

Analysis of iNOS mRNA

The expressions of iNOS was determined by semi-quantitative RT-PCR, using β-actin as an internal control. Primary peritoneal macrophages (6 $\times$ 10^6 cells per 100 mm culture dish) were pre-treated with tilianin (1, 10, and 100 μM) for 2 h, and stimulated with LPS (2 μg/ml) for 18 h. Total RNA was extracted from the macrophages using a TRIZOL (Gibco BRL, MD) reagent. The forward primer for mouse iNOS (1) 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3', and the reverse primer (2) 5'- GGG AGT AGC CTG TGT GCA CCT GGA A -3' define an amplicon of 311 bp. The forward primer for β-actin (1) 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', and the reverse primer (2) 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' define an amplicon of 349 bp. The densitometric analysis was performed with Image Analysis Software (Bio 1D Version 99, Vilber Lour-

mat, France) to determine the relative band density. The internal control was used to avoid saturation of PCR product.

Transient transfection assay

A spontaneously transfected mouse monocyte/macrophage cell (RAW264.7) was used because of their high transfection efficiency. Cells were plated in 6-well plates at 5×10^5 cells/well in DMEM (Gibco BRL, MD) containing 10% fetal calf serum. After growing at 37°C for 20 h, 3 μg of piNOS-Luc plasmids containing iNOS promoter (-1,029~ +159) (Lowenstein *et al.*, 1993) and luciferase reporter were transfected using FuGENE6 (Roche, Mannheim, Germany) according to the manufacturer's directions. For NF-κB activation, pNF-κB-Luc plasmid (5 × NF-κB; Stratagene, CA) was transfected to RAW264.7 cells. The control plasmid pCMVb (1 μg)

(Clontech, CA) was co-transfected to monitor the transfection efficiency. After 20 h of transfection, medium was changed and tilianin (1, 10, and 100 μM) was pretreated for 2 h before LPS (200 ng/ml) treatment. After additional 48 h cultures, cells were then harvested and luciferase activities were determined using the Dual Luciferase assay (Promega, WI). The luciferase activity was normalized for transfection efficiency after determining the β-galactosidase activity of the same sample. Each transfection was carried out in triplicate and experiments were repeated at least four times.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from cells by lysing them in buffer A [10 mM HEPES (pH 7.5), 1.5 mM MgCl$_2$, 10 mM KCl, and 0.1% Nonidet P-40]. Subsequently, the solution was centrifuged to pellet the
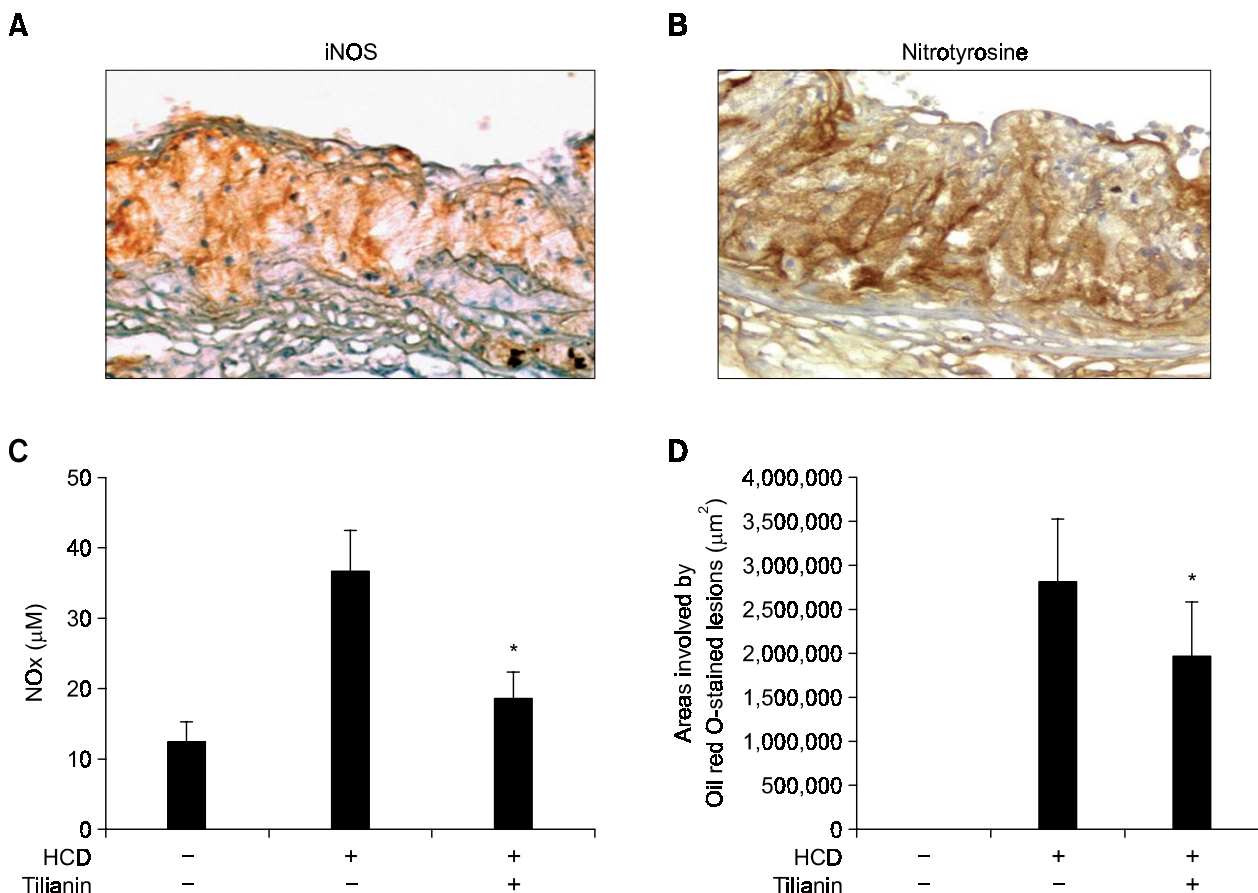

Figure 1. Localization of proatherogenic molecules in atherosclerotic lesions. iNOS (A, × 200) and nitrotyrosine (B, × 200) were detected by immunohistochemistry. Frozen sections of aortic segment were stained with polyclonal antibody for each molecule and detected using an ABC method. Section was then counterstained with Harris' hematoxylin. Control staining without primary antibodies did not show any significant staining in atherosclerotic lesion (data not shown). (C) Effect of tilianin on the plasma NO level in HCD-fed *Ldlr-/-* mice. The plasma was obtained from mice fed normal diet only, HCD only, and HCD supplemented with tilianin (0.01% wt/wt diet). (D) Oil red O stained aortic valve lesion areas were quantified by computer-associated morphometry. All results are shown as mean ± SD. * indicates $P < 0.01$ compared with HCD only group.

nuclei, and the collected nuclei was suspended in buffer B [20 mM HEPES (pH 7.5), 1.5 mM MgCl$_2$, 0.42 M NaCl, 0.2 mM EDTA, and 25% glycerol] and incubated on ice for 30 min. Following centrifugation, the nuclear extracts in the supernatant were harvested and protein concentrations were determined by Bradford method (Bio-Rad Laboratories, Inc., CA). For Electrophoretic mobility shift assays, 5 μg of nuclear protein was reacted with [^{32}P]-γATP-labeled NF-κB oligonucleotide (Promega, WI) in presence of 1 μg of poly (dI-dC) (Sigma). To check for specificity, antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology, CA) was added to the binding reaction. Nuclear extracts-oligonucleotide mixtures were then subjected to eletrophoresis on 5% nondenaturing polyacrylamide gels. Gels were then dried and visualized by autoradiography.

Statistics

The data are expressed as the mean ± SD. Statistical significance was determined by Student's *t* test.

Results

Localization of iNOS, nitrotyrosine in atheromatous plaques

We confirmed the iNOS expression in atheromatous plaque of *Ldlr-/-* mice fed HCD by immunohistochemistry using antibodies specific for mouse iNOS. Control staining without primary antibodies showed no staining in atherosclerotic lesion (data not shown). Localization of iNOS protein was prominent in the atheromatous thickened intima (Figure 1A). No immunoreactivity for nitrotyrosine was detected in normal aortic sinus area (data not shown). Nitrotyrosine, however, was easily detected in the corresponding lesion site in which prominent iNOS expression was detected (Figure 1B). These results indicate that iNOS expression in atheromatous plaques is associated with the formation of nitrotyrosine.

Effects of tilianin on plasma levels of NO and lesion formation

We next examined the effect of tilianin on the plasma level of NO. *Ldlr-/-* mice fed with HCD resulted in increased plasma level of NO$_x$, a stable oxidized product of NO. In mice fed with HCD supplemented tilianin, however, the NO$_x$ level was significantly decreased by about 50% (Figure 1C). In correspondence to the lowered NOx level, the
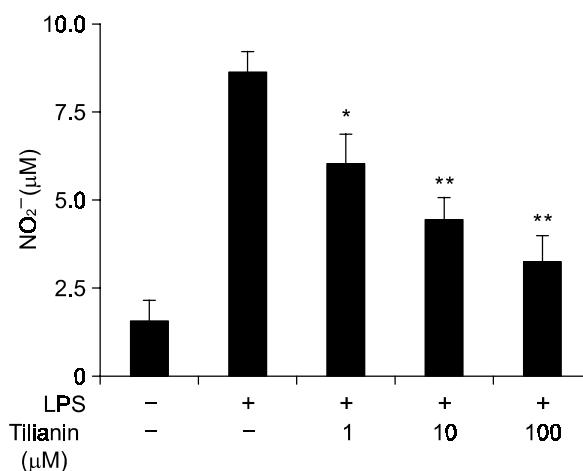

Figure 2. Effect of tilianin on NO production in LPS (2 μg/ml) activated peritoneal macrophage. Inhibition of NO production in LPS activated peritoneal macrophage by tilianin. The primary peritoneal macrophages from *Ldlr-/-* mice were incubated with tilianin (1, 10, 100 μM) for 2 h and then activated with LPS (2 μg/ml) for 18 h. The results are expressed as mean ± SD (*n* = 3). NO production was measured by Griess reaction. *,** indicates *P* < 0.05 and *P* < 0.01 compared with HCD only group, respectively.

lesions induced by HCD were decreased by the treatment of tilianin, similar to our previous study (Nam *et al.*, 2005) (Figure 1D).

Tilianin inhibits the NO production and iNOS expression under *in vitro* inflammatory condition

Since tilianin has anti-atherogenic effect, the decrease of serum NO level may reflect the consequence of the reduction of inflammatory cell accumulation in the lesion area. Thus, we investigate whether tilianin directly affect the production of NO and the expression of iNOS gene in peritoneal macrophages stimulated with LPS (2 μg/ml) in the presence of various concentrations of tilianin. LPS-induced increased NO production was decreased in concentration-dependent manner by tilianin (Figure 2). Moreover, increased expression of iNOS mRNA and protein in macrophages stimulated by LPS was markedly inhibited in a dose-dependent manner by the treatment of tilianin (Figure 3A, B and C, respectively). These results suggest that tilianin inhibits effectively the NO production by modulating iNOS expression under *in vitro* inflammatory condition.

Tilianin inhibits iNOS promoter activity and NF-κB activation

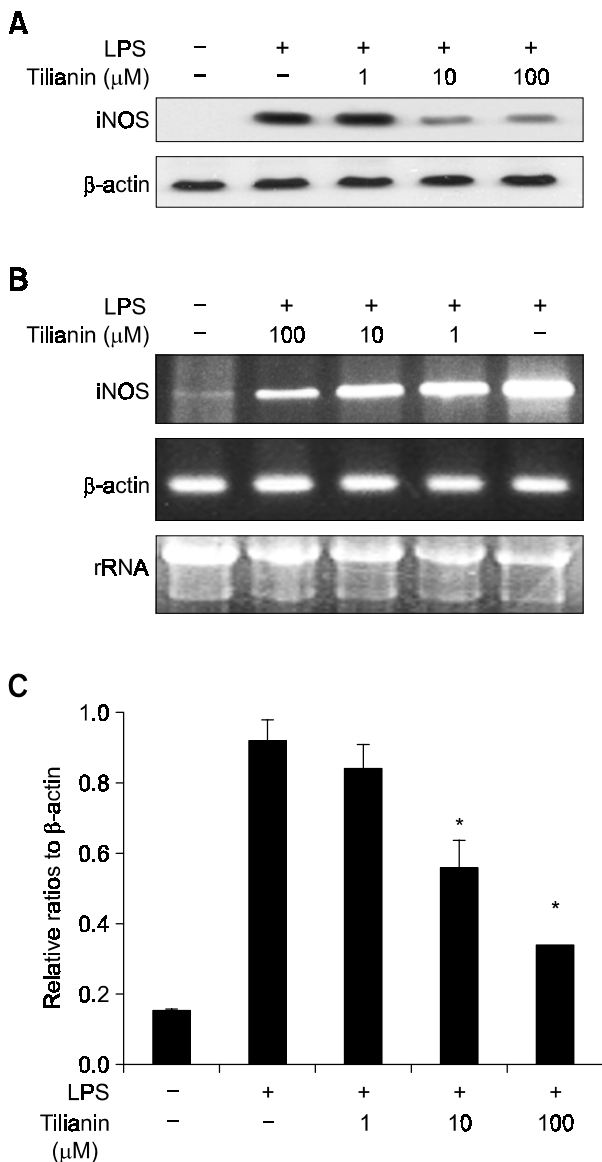We examined whether tilianin suppressed iNOS promoter activity. Transient transfection in the mouse

A

LPS	−	+	+	+	+
Tilianin (μM)	−	−	1	10	100

iNOS

β-actin

B

LPS	−	+	+	+	+
Tilianin (μM)	−	100	10	1	−

iNOS

β-actin

rRNA

C

Figure 3. Effect of tilianin on iNOS expression in LPS (2 μg/ml) activated peritoneal macrophage. (A) Inhibition of iNOS expression in peritoneal macrophage by tilianin. iNOS expression was determined by immunoblot analysis as described in Methods. (B) Determination of iNOS mRNA expression levels by semi-quantitative RT-PCR. (C) A graph showing relative iNOS mRNA level in B. The primary peritoneal macrophages from *Ldlr-/-* mice were incubated with tilianin (1, 10, 100 μM) for 2 h and then activated with LPS (2 μg/ml) for 18 h. The results are expressed as mean ± SD (*n* = 3). NO production was measured by Griess reaction. * indicates *P* < 0.05 compared with HCD only group, respectively.

macrophage cell line RAW264.7 cells was performed with a murine iNOS promoter construct (piNOS-Luc) by lipofection. Treatment of the transfected cells with LPS and IFN-γ resulted in a 15-fold increase in luciferase activity. This increase was suppressed in a dose-dependent manner by increasing concentrations of tilianin (Figure 4A). The expression of iNOS has been reported to be regulated by NF-κB activation (Barnes and Karin, 1997). Therefore, it is possible that tilianin inhibits NO production in peritoneal macrophages by inhibition of NF-κB activation. To investigate the roles of tilianin in NF-κB dependent gene transcription, we conducted a transient transfection assay using a DNA construct containing SV40 promoter, 5 repeats of the consensus NF-κB binding sequence, and the luciferase reporter gene. Stimulation of the transfectant with LPS resulted in an approximate 45-fold increase in luciferase activity, and this increase was inhibited dose-dependently by tilianin treatment (Figure 4B). These results indicate that tilianin inhibits NF-κB activation and may subsequently suppress iNOS and inflammatory cytokines of which expressions are NF-κB-dependent. Next, the effect of tilianin on NF-κB activation in peritoneal macrophages was evaluated by electrophoretic mobility shift assay. The nuclear extract from LPS-stimulated primary macrophages showed an increase in NF-κB-DNA binding activity, whereas any protein-oligonucleotide complex was not detected in unstimulated control cells. The binding activity was suppressed in a dose-dependent manner by the addition of tilianin (Figure 4C). Specificity of the DNA-protein interaction for NF-κB was demonstrated by the presence of antibody for the p65 subunit of NF-κB in the complex using a supershift assay (Figure 4C).

Discussion

We investigated the effect of tilianin upon inducible nitric oxide production *in vivo* and *in vitro*. We demonstrated that tilianin reduced the level of nitric oxide in plasma from *Ldlr-/-* mice fed with HCD and that tilianin suppressed the expression of iNOS gene in peritoneal macrophages isolated from *Ldlr-/-* mice. This compound also inhibited the transcriptional activation of iNOS promoter that has NF-κB binding element. Thus, these results provide the first evidence that tilianin inhibit iNOS expression and NO production, suggesting a possible anti-atherogenic mechanism of tilianin in hyperlipidemic model mice.

Nitric oxide produced by iNOS inhibits the proliferation and induces apoptosis in vascular endothelial cells (Cornwell *et al.*, 1994; Fukuo *et al.*, 1996). It also produces the powerful oxidant peroxynitrite ($ONOO^-$) by interacting with the reactive oxygen superoxide (Beckman and Koppenol, 1996). Peroxynitrite has been strongly implicated as a
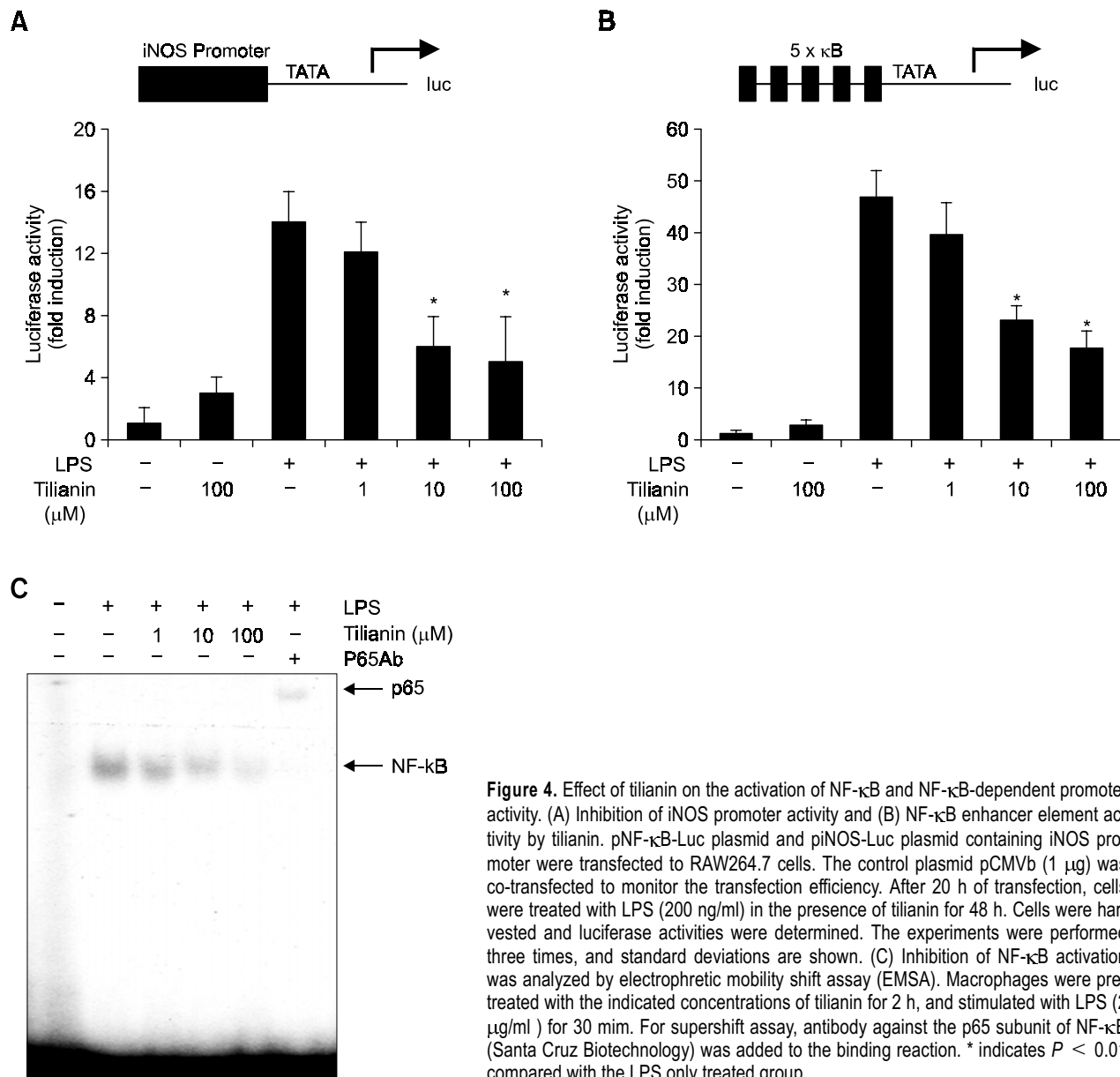
A

B

C

Figure 4. Effect of tilianin on the activation of NF-κB and NF-κB-dependent promoter activity. (A) Inhibition of iNOS promoter activity and (B) NF-κB enhancer element activity by tilianin. pNF-κB-Luc plasmid and piNOS-Luc plasmid containing iNOS promoter were transfected to RAW264.7 cells. The control plasmid pCMVb (1 μg) was co-transfected to monitor the transfection efficiency. After 20 h of transfection, cells were treated with LPS (200 ng/ml) in the presence of tilianin for 48 h. Cells were harvested and luciferase activities were determined. The experiments were performed three times, and standard deviations are shown. (C) Inhibition of NF-κB activation was analyzed by electrophretic mobility shift assay (EMSA). Macrophages were pretreated with the indicated concentrations of tilianin for 2 h, and stimulated with LPS (2 μg/ml) for 30 mim. For supershift assay, antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology) was added to the binding reaction. * indicates $P < 0.01$ compared with the LPS only treated group.

cytotoxic effector molecule contributing to cellular damage and promoting the formation of atherosclerotic lesion (Wilcox *et al.*, 1997). However, there are several conflicting papers regarding the effect of iNOS inhibition on atherosclerotic lesion formation using genetically engineered mice. When fed with normal diet, iNOS/ApoE double knockout mice did not have any different lesion compared to ApoE littermate controls (Knowles *et al.*, 2000). Furthermore, iNOS knockout mice fed with normal diet have hypertension, a two fold increase in plasma cholesterol level, and aortic atherosclerotic lesion (Ihrig *et al.*, 2001). On the contrary, when fed with atherogenic diet, the lesion size was decreased in iNOS/

ApoE double knockout mice compared to littermate ApoE-/- controls (Detmers *et al.*, 2000; Kuhlencordt *et al.*, 2001). These findings suggest that excessively elevated plasma lipid level induced by atherogenic diet discloses the proathergenic potential of iNOS. In the present study, we investigated the possibility that tilianin would prevent atherosclerosis induced by HCD. Our data showed that iNOS expression and nitrotyrosine formation were detected in the atheromatous lesion, and tilianin effectively reduced NO synthesis *in vitro* or *in vivo*, suggesting that the inhibitory effect of tilianin on the production of NO and peroxynitrite is plausible mechanism responsible for its antiatherogenic activity.

We further demonstrated that iNOS expression was significantly lowered by treatment of tilianin. Promoter region of iNOS contains NF-κB binding sequence, and NF-κB activation is required for iNOS gene expression (Lowenstein *et al.*, 1993). We examined the role of tilianin in iNOS expression and its promoter activity *in vitro*. Transient transfection with reporter genes with NF-κB binding elements to RAW264.7 cells showed clearly that tilianin inhibited significantly NF-κB-dependent iNOS expression, which is responsible for reduced NO production. In conclusion, tilianin significantly inhibit NO production and iNOS expression through the inhibition of NF-κB dependent transcription. Thus, the inhibition of NO production could be a novel possible mechanism of the anti-atherogenic potential of tilianin. And, it also implies that tilianin has potential as an anti-inflammatory agent.

Acknowledgement

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