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

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Abbreviations: HUVEC, human umbilical endothelial cell; Ldlr, low density lipoprotein receptor; NO, nitric oxide; NOS, nitric oxide synthase; VCAM-1, vascular cell adhesion molecule-1

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 production 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\textsuperscript{-}) (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, 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) dilutions, then the reactivities were detected using ABC kit (Vector Laboratory, CA) and a rabbit anti-mouse iNOS antibody (Santa Cruz Biotechnology, CA) and a rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, NY) 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 (NOx) 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 × 10⁶ 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 CAT TGT 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-
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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 $5 \times 10^5$ 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) (Löwenstein 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; Stratagen, 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 tiliannin (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 MgCl₂, 10 mM KCl, and 0.1% Nonidet P-40]. Subsequently, the solution was centrifuged to pellet the...
nuclei, and the collected nuclei was suspended in buffer B [20 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 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 [32P]-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 electrophoresis 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 NOx, a stable oxidized product of NO. In mice fed with HCD supplemented tilianin, however, the NOx level was significantly decreased by about 50% (Figure 1C). In correspondence to the lowered NOx level, the 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) 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 ** indicates P < 0.01 compared with HCD only group, respectively.

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 ** indicates P < 0.01 compared with HCD only group, respectively.

Tilianin inhibits iNOS promoter activity and NF-κB activation
We examined whether tilianin suppressed iNOS promoter activity. Transient transfection in the mouse
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macrophage cell line RAW264.7 cells was performed with a murine iNOS promoter construct (pNOS-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 tiliarin (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 tiliarin inhibits NO production in peritoneal macrophages by inhibition of NF-κB activation. To investigate the roles of tiliarin 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 tiliarin treatment (Figure 4B). These results indicate that tiliarin inhibits NF-κB activation and may subsequently suppress iNOS and inflammatory cytokines of which expressions are NF-κB-dependent. Next, the effect of tiliarin 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 tiliarin (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 tiliarin upon inducible nitric oxide production in vivo and in vitro. We demonstrated that tiliarin reduced the level of nitric oxide in plasma from Ldlr−/− mice fed with HCD and that tiliarin 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 tiliarin inhibit iNOS expression and NO production, suggesting a possible anti-atherogenic mechanism of tiliarin 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
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 controls (Detmers et al., 2000; Kuhlencordt et al., 2001). These findings suggest that excessively elevated plasma lipid level induced by atherogenic diet discloses the proatherogenic 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.

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 pNOS-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 electrophoretic mobility shift assay (EMSA). Macrophages were pre-treated with the indicated concentrations of tilianin for 2 h, and stimulated with LPS (2 μg/ml) for 30 min. 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.
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.

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