Anti-inflammatory effect of lovastatin is mediated via the modulation of NF-κB and inhibition of HDAC1 and the PI3K/Akt/mTOR pathway in RAW264.7 macrophages

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Abstract. Lovastatin is a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor that is clinically used for the prevention of cardiovascular diseases. Although it has been reported that lovastatin has anti-inflammatory properties in several studies, how lovastatin regulates the inflammation is still unclear. To evaluate the effect of lovastatin on nitric oxide production (NO) in RAW264.7 macrophages, NO production assay was performed. Also, cell viability was measured to confirm cytotoxicity. Level of tumor necrosis factor-α (TNF-α) transcription was measured by reverse transcription polymerase chain reaction (RT-PCR) from total RNA in RAW264.7 cells. Western blot analysis and immunofluorescence staining were used to investigate the regulation of lovastatin on the expression, phosphorylation, and nuclear translocation of cellular proteins. The results of the present study revealed that lovastatin reduced nitric oxide production via the reduction of inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. The mRNA level of TNF-α was reduced in presence of lovastatin. In addition, lovastatin downregulated histone deacetylase 1 (HDAC1), resulting in the accumulation of acetylated histone H3 and heat shock protein 70. Furthermore, the expression of phosphoinositide 3-kinase catalytic subunits α and β was reduced under lovastatin treatment, and the phosphorylation of Akt and mammalian target of rapamycin was consequently inhibited.

Keywords: lovastatin, anti-inflammation, nuclear factor-κB, histone deacetylase, phosphoinositide 3-kinase/Akt

Lovastatin also inhibited the phosphorylation of inhibitor of nuclear factor (NF)-κB and the translocation of NF-κB into the nucleus. Therefore, the present study demonstrates that lovastatin inhibits the expression of pro-inflammatory mediators, including iNOS and TNF-α, through the suppression of HDAC1 expression, PI3K/Akt phosphorylation and NF-κB translocation in LPS-stimulated RAW264.7 macrophage cells.

Introduction

Inflammation is a defense response against infection, tissue injury or noxious stimuli. In the inflammatory process, inflammatory products such as nitric oxide (NO) and pro-inflammatory cytokines serve important roles (1). In particular, NO is a crucial mediator for the amplification of the inflammatory response. NO is generated from L-arginine by inducible nitric oxide synthase (iNOS). NO has various biological functions in mammalian cells, including microbial immunity, vasodilation and the modulation of cell signaling (2). However, the excessive production of NO may cause damage to normal tissues and exacerbate inflammatory diseases (3). The expression of iNOS and other inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), is regulated by several transcription factors, one of which is nuclear factor-κB (NF-κB) (4). Therefore, the regulation of NO production in an excessive inflammatory response may facilitate the improvement of the inflammatory symptoms.

Previous studies have shown that NF-κB is regulated by several mechanisms, including histone acetylation and the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway (5,6). The former mechanism, histone acetylation, is a post-transcriptional modification process that is involved in the regulation of gene transcription. This process is highly reversible and widespread in eukaryotes. Lysine residues in histones are a target of histone acetylation. Histone acetyltransferases and histone deacetylases (HDACs) are enzymes that regulate histone acetylation and deacetylation, respectively (7). One of the proteins that is regulated by histone acetylation and deacetylation is heat shock protein 70 (HSP70) (8). It has

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been revealed that the accumulation of HSP70 in the cytoplasm prevents the translocation of cytosolic NF-κB into the nucleus (8). In the latter mechanism of NF-κB regulation, PI3K/Akt/mTOR signal transduction. PI3K catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate, which then phosphorylates Akt. The phosphorylated Akt mediates the phosphorylation of mTOR, and the phosphorylated mTOR encourages the translocation of NF-κB into the nucleus (9,10).

Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) used clinically for the prevention of cardiovascular diseases via the lowering of cholesterol levels. Commercially, lovastatin is obtained by the fermentation of *Pleurotus ostreatus*, but several edible mushrooms, such as *Pleurotus ostreatus*, are reported to produce high quantities of lovastatin (11). Studies have demonstrated that lovastatin also has anticancer and anti-inflammatory effects in vitro and in vivo (12,13). The chemical structure of lovastatin is presented in Fig. 1 (12).

The precise mechanisms associated with the anti-inflammatory effects of lovastatin have not been fully elucidated. Therefore, in the present study, the anti-inflammatory effects of lovastatin were investigated and its molecular mechanism was explored, focusing on HDAC1, HSP70 and the PI3K/Akt/mTOR signaling pathway in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. The results should indicate whether lovastatin has potential as an inflammatory suppressor, and may support the further clinical application of lovastatin.

**Materials and methods**

**Cell culture and reagents.** RAW264.7 murine macrophage cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (100 U/ml)/streptomycin (100 µg/ml). The cells were incubated with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin. The medium in each well was then discarded, and 100 µl DMEM containing 10% FBS was added to each well followed by 10 µl EZ-cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service, Gyegngi, Korea) and the cells were incubated for 3 h. Following this, the optical density was measured at 460 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

**Western blot analysis.** RAW264.7 cells were treated with LPS only or following pretreatment with lovastatin as described above prior to the western blotting of cell lysates. The cells were extracted using cell lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.5% NP-40, 1% Triton X-100, 1% deoxycholate and 0.1% sodium dodecyl sulfate (SDS)] and a cocktail of proteinase inhibitors (phenylmethylsulfonyl fluoride, EDTA, aprotinin, leupeptin and progesterin A; Intrion Biotechnology, Inc., Seongnam, Korea). For the preparation of nuclear extracts, the cultured cells were harvested and lysed using NE-PER® Nuclear and Cytoplasmic Extraction reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The protein concentration in the cell lysates was measured with a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

**Reverse transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted from RAW264.7 cells using 2-mercaptoethanol (Sigma-Aldrich; Merck KGaA) and isolated using PureLink mini kit (Qiagen, Venlo, Netherlands). A nanodrop (MÉCASYS, Daejeon, Korea) was used for quantifying the concentrations of total RNA. RNA (1 µg) was synthesized to cDNA with cDNA kit (Genetbio, Daejeon, Korea) and cDNA synthesis was performed at 42°C for 1 h and then at 94°C for 5 min. Genes encoded in 1 µg cDNA were amplified by PCR using specific primers as follows: TNF-α forward, 5'-CCC CTC AGC AAA CCA CCA AGT-3' and
reverse, 5'-CTT ggg CAg ATT gAC CTC AgC-3'; gAPDH forward, 5'-AAC TTT gg C ATT g Tg g AA gg -3' and reverse, 5'-CAC ATT ggg ggT Agg AAC AC-3'. PCR was performed under the following conditions: denaturation (92˚C, 1 min), annealing (57˚C, 30 sec), extension (72˚C, 30 sec), and 23 cycles. Amplified PCR products were observed on 2% agarose gel with ethidium bromide. The intensity of bands was measured by Image J software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining and confocal microscopy of NF-κB. RAW264.7 cells were seeded on coverglass-bottom dishes (SPL Life Sciences, Pocheon, Korea) and pretreated with 10 µM lovastatin for 1 h. The cells were then stimulated with incubation with LPS (1 µg/ml) for 24 h. Following incubation, the cells were stained using 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at 37˚C, washed with PBS and fixed with 4% formaldehyde (Junsei Chemical Co., Ltd., Tokyo, Japan) for 15 min at room temperature in the dark. The cells were blocked with blocking solution (5% normal rabbit serum and 0.3% Triton X-100) for 1 h in the dark at room temperature. The normal rabbit serum was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and Triton X-100 was purchased from Sigma-Aldrich (Merck KgaA). The cells were incubated with the anti-NF-κB primary antibody (dilution, 1:2,000; Cell Signaling Technology, Inc.) at 4˚C overnight. Following the reaction, the cells were washed with PBS and then treated with anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor® 488 conjugate; dilution, 1:2,000) for 1 h at room temperature. The cells were washed with PBS and Prolong Gold Anti-fade Reagent® (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the slide at room temperature. The cells were observed using a Carl Zeiss LSM 710 confocal laser scanning microscope (Zeiss GmbH, Jena, Germany).

Statistical analysis. To determine the statistical significance of the data, GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used. The results are expressed as the mean ± standard deviation. Every experiment was performed in triplicate. One-way analysis of variance with post hoc Dunnett's multiple comparison tests were used to assess differences among the untreated, LPS only and lovastatin-treated groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Viability of RAW264.7 cells following lovastatin treatment. The viability of the RAW264.7 macrophage cells was measured using the WST-1® assay. The cells were treated with 1, 10, 50 or 100 µM lovastatin with 1 µg/ml LPS. Lovastatin exhibited no significant effect on cell viability at concentrations ≤50 µM, but 100 µM lovastatin significantly inhibited the viability of the cells (Fig. 2). On the basis of the results of the cell viability assay, ≤50 µM lovastatin was used in further experiments to exclude the cytotoxic effects of lovastatin on RAW264.7 cells.

Lovastatin decreases NO production, iNOS and TNF-α expression in RAW264.7 cells. NO production was determined using Griess reagent in RAW264.7 cells treated with LPS alone, or LPS and lovastatin. When cells were treated with LPS only, the level of NO production was increased. However, when the RAW264.7 cells were also treated with 10 or 50 µM lovastatin, NO production was significantly decreased compared with that of the LPS-only treated cells (Fig. 3A). Since NO production was downregulated by 10 and 50 µMlovastatin but not 10 µM, it was postulated that 10 and 50 µM lovastatin may alter iNOS expression. Western blotting revealed that lovastatin decreased the expression of iNOS in an apparently dose-dependent manner, compared with that in the cells treated with LPS only (Fig. 3B). The mRNA level of TNF-α was also compared between the RAW264.7 cells treated with LPS alone and those
Lovastatin prevents the nuclear translocation of NF-κB in LPS-stimulated RAW264.7 cells. As the aforementioned results show that iNOS and TNF-α expression were reduced at the protein and mRNA levels, respectively, it was hypothesized that lovastatin may affect the activation of transcription factors. Among various transcription factors, NF-κB is well-studied for its broad participation in the transcription of inflammatory mediators, including iNOS and TNF-α (14). To verify the regulatory effect of lovastatin on NF-κB activation, the nuclear expression of NF-κB was evaluated. The expression of NF-κB in nuclear extracts from the LPS-stimulated RAW264.7 cells was decreased by lovastatin, and the reduction appeared to be dose-dependent. Also, the expression of IkBα, which inhibits the nuclear translocation of NF-κB (15), was increased while the phosphorylation of IkBα was decreased, and the reduction in phosphorylation appeared to be a dose-dependent (Fig. 4A). When the cells were subjected to immunofluorescence staining (Fig. 4B), the expression of NF-κB (green) was detected in the nucleus (blue) as well as the cytosol of cells when the cells were treated with LPS only. However, when the RAW264.7 cells were treated with LPS plus 10 µM lovastatin, the expression levels of nuclear NF-κB (green) were decreased. The results demonstrate that lovastatin hinders LPS-induced NF-κB translocation from the cytosol into the nucleus, indicating that lovastatin may thereby suppress the inflammatory mediators, including iNOS and TNF-α, transcribed by NF-κB.

Lovastatin induces HSP70 accumulation via HDAC1 inhibition. Previous studies have indicated that HDAC1 mediates the exposure of the HSP70 promoter region through the acetylation of histone H3, and that HSP70 upregulation is able to block NF-κB translocation (16,17). In the present study, HDAC1 expression was markedly decreased by lovastatin, and the reduction appeared to be dose-dependent (10 and 50 µM). However, lovastatin treatment increased the acetylation of histone H3 at lysine 9. The expression and accumulation of HSP70 was increased when the RAW264.7 cells were treated with LPS in combination with 10 or 50 µM lovastatin (Fig. 5). Therefore, these results indicate that lovastatin regulates the expression of HSP70 by inducing the downregulation of HDAC1, which leads to the increased acetylation of histone H3 at the lysine 9 residue.

Lovastatin inhibits the PI3K/Akt/mTOR signaling pathway. In the present study, the PI3K/Akt/mTOR signaling pathway in LPS-stimulated macrophages appeared to be inhibited by the presence of lovastatin. The protein levels of the PI3K 110α and p110β catalytic subunits were increased when the cells were treated with LPS only; however, when the cells were treated with 10 or 50 µM lovastatin prior to LPS, the expression of the two PI3K subunits was decreased. Likewise, the phosphorylation of Akt at serine 473 residue and mTOR at serine 2481 residue were decreased by lovastatin in a comparable manner. However, lovastatin did not alter the expression of Akt and mTOR in the whole-cell lysates (Fig. 6). Therefore, these results suggest that lovastatin exerts regulatory effects on the PI3K/Akt/mTOR signaling pathway in LPS-stimulated RAW264.7 cells.

Discussion

Inflammation is an important innate immune response that protects the host from antigens. However, the overexpression of pro-inflammatory mediators, such as NO and pro-inflam-
matory cytokines, may occur as an aberrant inflammatory response (18,19). Macrophages serve pivotal roles in inflammation through the production of pro-inflammatory mediators and the regulation of inflammatory cells (18,20).

Lovastatin has been prescribed as a drug for the treatment of cardiovascular diseases for many years, and it has also been reported that lovastatin is able to inhibit tumor growth and inflammation (11,13,21). Previous studies have demonstrated that the inhibition of HDAC affects inflammation via various signaling pathways (22,23). In addition, many studies have revealed that the PI3K/Akt signaling pathway is associated with the inflammatory response. PI3K/Akt signal transduction acts upstream of NF-κB activation (24-26). Specifically, the inhibition of PI3K p110α and p110β subunits is reported to have a therapeutic function in the treatment of chronic inflammatory diseases (27). Therefore, in the present study, whether lovastatin reduces inflammation by the downregulation of HDAC and PI3K/Akt in LPS-stimulated RAW264.7 macrophage cells was investigated.

NO serves a key role in inflammation and it is used for the evaluation of macrophage activation (28). In the present study, whether lovastatin reduces inflammation by the downregulation of HDAC and PI3K/Akt in LPS-stimulated RAW264.7 macrophage cells was investigated.

Figure 4. Inhibition of NF-κB translocation into the nucleus and changes in IkBα expression and phosphorylation in LPS-stimulated RAW264.7 macrophages induced by treatment with lovastatin. The cells were pretreated with the indicated concentrations of lovastatin for 1 h prior to treatment with LPS (1 µg/ml) for 24 h, or were treated with LPS alone. (A) Protein expression levels of NF-κB were determined by the western blot analysis of nuclear extracts. (B) Immunofluorescence staining and confocal microscopy was used for observing NF-κB (green) translocation into the nucleus (DAPI; blue) (scale bar, 20 µm). NF-κB, nuclear factor-κB; IkBα, inhibitor of NF-κBα; p, phospho; LPS, lipopolysaccharide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole.
Lovastatin at the concentrations 10 and 50 µM decreased NO production without cytotoxicity in LPS-stimulated RAW264.7 cells. This result is consistent with the downregulation of iNOS expression that was also observed in response to lovastatin treatment in the present study, since iNOS promotes the production of NO (29). Another well-known target of anti-inflammatory treatment is TNF-α (30), the expression of which was decreased at the transcriptional level by lovastatin in the present study. Therefore, these data suggest that lovastatin regulates inflammation by decreasing NO production and TNF-α expression.

NF-κB is associated with the transcription of numerous pro-inflammatory factors, including iNOS and TNF-α (31). When activated, NF-κB is phosphorylated and translocated into the nucleus (32). However, the present study demonstrated through western blot analysis and immunofluorescence staining that the translocation of NF-κB was attenuated by lovastatin in LPS-stimulated RAW264.7 cells. Previously, studies have shown that NF-κB participates in the expression of iNOS and TNF-α (33-35). IκBα binds to NF-κB and suppresses its translocation; when IκBα is phosphorylated and degraded by proteasomes, NF-κB is activated and translocates into the nucleus (36). The western blotting results in the present study indicate that the expression of IκBα was increased and its phosphorylation was decreased in the presence of lovastatin. Thus, the lovastatin-induced reduction of iNOS and TNF-α expression may result from the inhibition of NF-κB translocation into the nucleus.

NF-κB activation is also regulated by HDAC1 expression. When HDAC1 expression is decreased, histone H3 acetylation at the lysine 9 residue is facilitated. The promoter region of the HSP70 gene is then exposed, which promotes HSP70 transcription (8). The accumulation of HSP70 in the cytoplasm leads to inhibition of the translocation of NF-κB from the cytoplasm into the nucleus (17). On the basis of previous studies, it was hypothesized that reduction of NF-κB translocation into the nucleus is associated with decreased HDAC1 expression and increased HSP70 expression. The western blotting results in the present study reveal that HDAC1 expression was downregulated by lovastatin in LPS-stimulated RAW264.7 cells. Also, histone H3 acetylation at lysine 9 and HSP70 expression was upregulated by lovastatin (10 and 50 µM) in the LPS-stimulated RAW264.7 cells. These results support the hypothesis that the inhibitory effect of lovastatin on HDAC1 disturbs the translocation of cytosolic NF-κB into the nucleus in LPS-stimulated RAW264.7 macrophages.

Although lovastatin may reduce NF-κB nuclear translocation via the induction of HSP70, the possibility that lovastatin influences other signal transduction pathways to regulate NF-κB activation in the cytoplasm was also considered. It has been reported that PI3K/Akt/mTOR signaling transduction contributes to NF-κB translocation in the inflammatory response; PI3K phosphorylates Akt at serine 473, which induces NF-κB translocation (9). This regulatory function of the PI3K/Akt signaling pathway on NF-κB translation occurs through the phosphorylation of mTOR, downstream of Akt, at the serine 2481 residue (37). In the present study, western blotting revealed that the expression of PI3K p110α and p110β catalytic subunits in LPS-stimulated RAW264.7 cells was decreased by 10 and 50 µM lovastatin. In addition, the phosphorylation of PI3K and Akt was downregulated by lovastatin, resulting in reduced levels of mTOR expression in the LPS-stimulated RAW264.7 cells. This is likely to prevent the translocation of cytosolic NF-κB into the nucleus in the LPS-stimulated RAW264.7 cells.

In conclusion, the results of the present study indicate that the downregulation of HDAC1 expression and inhibition of the PI3K/Akt/mTOR signaling pathway by lovastatin results in the inhibition of cytosolic NF-κB translocation into the nucleus in LPS-stimulated RAW264.7 macrophages. Furthermore, the inhibitory effect of lovastatin on NF-κB activation induces an anti-inflammatory response in LPS-stimulated RAW264.7 macrophage cells through suppression of the expression of iNOS and TNF-α. These observations suggest that lovastatin is a potential therapeutic agent for the amelioration of inflammatory diseases via the regulation of macrophages.
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