Inhibition of NF-κB activity in rabbit vascular smooth muscle cells by lovastatin

LUAN Zhao-Xia,[1] LAN Xiao-Li[2]

([1]The Third Affiliated Hospital, China Medical University, Shenyang 110022;

[2] The Second Affiliated Hospital, China Medical University, Shenyang 110003)

Abstract Nuclear factor NF-κB is believed to play an important role in regulating the production of matrix metalloproteinases (MMPs), which induce atherosclerosis, restenosis and plaque rupture. We incubated rabbit vascular smooth muscle cells(RVSMCs)with 5 μmol/L lovastatin in the presence of IL-1-α and $PDGF_{BB}$ (20 μg/L, respectively) to study whether lovastatin inhibited NF-κB binding activity induced by IL-1 and PDGF. The NF-κB activity was detected by electrophoretic mobility shift assay (EMSA); MMP-1 and MMP-3 were measured by western blotting; and MMP-9 was detected by zymography. The result showed that lovastatin strongly reduced NF-κB activity upregulated by IL-1 combined with PDGF, and lovastatin also dose-dependently inhibited the expression of MMP-1, -3 and -9 induced by IL-1 and PDGF. It suggested that the beneficial effects of statins may extend to mechanisms beyond cholesterol reduction.

Keywords NF-κB, Matrix metalloproteinases, Lovastatin, Atherosclerosis

CLC numbers $Q513^{+}.1$, R541.4

1 Introduction

Hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely used to treat hyperlipidaemia, and their use is associated with significant reduction of adverse coronary events.[1-4] Recent studies, both in vitro and in vivo, have suggested that the beneficial effects of statins may extend to mechanisms beyond cholesterol reduction. These pleiotropic effects of statins include improving or restoring endothelial function, inhibiting proliferation and migration of SMCs, decreasing vascular inflammation and, importantly, enhancing the stability of atherosclerotic plaques.[5,6] We therefore investigated whether lovastatin modulates NF-κB binding activity in cultured rabbit vascular smooth muscle cells (VSMCs), which play an important role in regulating the production of matrix metalloproteinases(MMPs) that induce atherosclerosis, restenosis and plaque rupture.[7,8]

2 Methods

2.1 Reagents

Human recombinant IL-1-α and human recombinant $PDGF_{BB}$ were purchased from R&D System. Sheep polyclonal anti-rabbit MMP-1 and MMP-3 antibodies were a generous gift from Dr G. Murphy, University of East Anglia, UK. Other reagents were purchased from Sigma Company unless otherwise stated.

2.2 Tissue culture

Primary culture of rabbit aortic smooth muscle cells was prepared by modifications of the explant technique, as previously described in detail.[7,9] Explants were maintained in complete medium composed of DMEM (Gibco Company) containing penicillin-streptomycin (100 units/mL and 100 mg/L, respectively), 8 mmol L-glutamine and 15% foetal bovine serum (FBS, Advanced Protein Products, UK). After 10~14 days, cells were subcultured by trypsin/EDTA treatment. Cells between passage one and passage three were plated at a density of 1×10^{6} cells/75 cm^2 flasks for nuclear extracts or 2×10^{5} cells/well into 6-well culture plates for zymography and western blotting. For all experiments, sub-confluent cells were rendered quiescent by incubation in serum-free

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DMEM supplemented with 0.25% (*V*/*V*) lactalbumin hydrolysate (Gibco BRL, Paisley, UK) for 3 days. Cultures were then exposed to fresh serum-free medium containing the appropriate concentration of the agent under investigation for 48 h.

2.3 Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from 2×10^6 rabbit vascular smooth muscle cell nuclei as previously described.[7,10,11] Oligonucleotides corresponding to the consensus NF-κB element (5'-AGT GGG GAC TTT CCC AGG C-3') (Promega) were annealed by heating in a boiling water bath for 5 min and allowing to slowly cool to room temperature, and 4 pmol of oligonucleotide was 5' end labeled with 1.48 MBq[γ - ^{32}P] ATP using T4 polynucleotide kinase at 37ºC for 30 min. The labeled oligonucleotide was then purified from unlabeled one using Nuc-trap columns (Amersham) in accordance with the manufacturer's instructions. Binding reactions (20 μL) containing 2 μg poly (dI-dC), 10 mmol HEPES (pH 7.9), 50 mmol NaCl, 0.5 mmol DDT, 2.5 mmol EDTA, 7 mmol $MgCl_2$, 4% glycerol, and 5 μg nuclear extract were incubated with 20,000 cpm of ^{32}P-labeled oligonucleotides at 37ºC for 15 min. Complexes were separated on 20% nondenaturing polyacrylamide gels, and the dried gels were then exposed to X-ray film at -80ºC for 72 h to be visualized by autoradiography. Bands were quantified in the linear range by densitometry with a GS 690 Image Analysis software system (Bio-Rad).

2.4 Zymography for MMP-9

Gelatinase activity was detected in conditioned media as previously described.[7,9] Briefly, 15 μL aliquots of conditioned media diluted 1:1 with nonreducing Laemmli sample buffer (2×) were electrophoresed at 4°C in 7.5% SDS-polyacrylamide gels containing 2 g/L gelatin derived from calf skin collagen. After removal of SDS gelatinase activity was revealed by overnight incubation at 37°C and stained with 0.1% Coomassie Brilliant Blue. Zymograms were quantified in the linear range by densitometry with a GS 690 Image Analysis software system (Bio-Rad).

2.5 Western blotting for MMP-1 and MMP-3

Western blotting was performed on conditioned media samples concentrated 10-fold by ultrafiltration using Amicon 10 centrifugal concentrators (Amicon, Stonehouse,Gloucestershire, UK). Samples were separated by 8% SDS-PAGE and blotted onto a Hybond-nitrocellulose membrane (Amersham) using a semidry blotting apparatus. Blocking of non-specific binding and dilutions of the primary (40 mg/L) anti-MMP1 or anti-MMP3 and secondary antibodies (1:2000, DAKO) used 5% skimmed milk powder /Tris-buffered saline /0.2% Tween 20. Protein was visualized by using an enhanced chemiluminescence system (ECL, Amersham). Bands were quantified by densitometry with a GS 690 Image Analysis software system (Bio-Rad).

2.6 Cell viability and proliferation studies

Viable cell numbers were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2, -5-diphenyltetrazolium bromide (MTT) assay (Sigma) according to the manufacturer's instruction.

2.7 Statistical analysis

All data are presented as mean±SEM of duplicate samples from at least 3 independently performed experiments. Differences were taken as statistically significant at $p<0.05$.

3 Results

In rabbit VSMCs, the NF-κB binding activity was very low in the absence of exogenous stimuli, but was significantly enhanced by a combination of IL-1-α (20 μg/L) and $PDGF_{BB}$ (20 μg/L). Lovastatin (5 μmol/L) strongly lowered the NF-κB binding activity induced by IL-1-α and $PDGF_{BB}$ (Fig.1).

The MMP-2 secretion was constitutive in rabbit VSMCs, while secretion of MMP-1, -3 and -9 was strongly increased from undetectable levels by a combination of IL-1-α (20 μg/L) and $PDGF_{BB}$ (20 μg/L) (Fig.2A). Lovastatin dose-dependently decreased MMP-1, -3 and -9 secretion induced by IL-1-α and $PDGF_{BB}$ (Fig.2B) in rabbit VSMCs.

There was no significant difference in viable cell

numbers among rabbit VSMCs cultures without or with different dose lovastatin, as determined by MTT assay (data not shown).

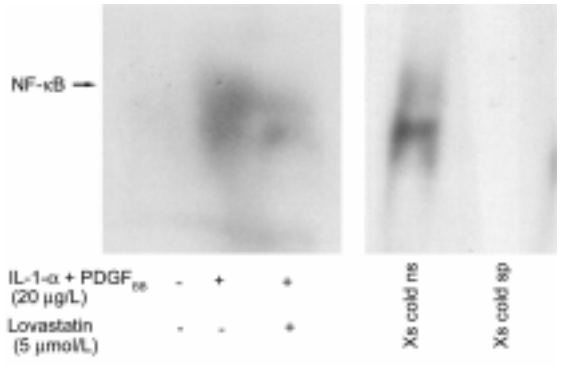

Fig.1 Lovastatin inhibited NF-κB activity upregulated by IL-1-α and $PDGF_{BB}$. Incubation of rabbit $VSMC_S$ with IL-1-α and $PDGF_{BB}$ (20 μg/L, respectively) for 48 h strongly raised NF-κB binding activity. Incubation of rabbit $VSMC_S$ with 5 μmol/L lovastatin for 48 h in the presence of IL-1-α and $PDGF_{BB}$ significantly reduced NF-κB binding activity upregulated by IL-1-α and $PDGF_{BB}$. NF-κB binding was inhibited by 100-fold excess (×s) specific (cold sp) unlabeled oligonucleotides, but not 100-fold non-specific (cold ns) unlabled oligonucleotides.

4 Discussion

The binding activity of NF-κB is negatively regulated by IκB proteins, which interact with NF-κB dimmers in the cytoplasm and inhibit entry into the nucleus. Exogenous stimulation, such as IL-1-α and TNF-α, induces phosphorylation and rapid proteolytic degradation of IκB, allowing NF-κB dimmers to translocate to the nucleus and activiate NF-κB.

HMG-CoA reductase catalyses the conversion of HMG-CoA to mevalonate, the precursor of isoprenoids. Treatment with lovastatin may cause mevalonate starvation inside VSMCs. Mevalonate metabolism yields a series of isoprenoid compounds, including squalene and geranlygeranlyphosphorate (GGPP). GGPP serves as important lipid attachment for the posttranslational modification of Rho GTPase family, whose translocation from cytoplasm to plasma membrane is dependent on geranylgeranylation.[5] Rho are small GTP-binding proteins that cycle between the inactive GDP-bound state and active GTP-bound state.[12] The Rho-like GTPases have been implicated in the activation of NF-κB. Evidence has been provided that the Rho GTPases, including Rho, Rac and Cdc42, mediate phosphorylation of IκB and cause translocation of the dimmers to the nucleus.[13,14]

NF-κB is an important transcription factor that regulates the transcription of MMP-1, -3 and -9 which play important role in atherosclerosis, restenosis and plaque rupture. Our study showed that lovastatin decreased the secretion of MMP-1, -3 and -9 stimulated by IL-1-α and $PDGF_{BB}$ dose-dependently. It suggested that the beneficial effects of statins may extend to mechanisms beyond cholesterol reduction.

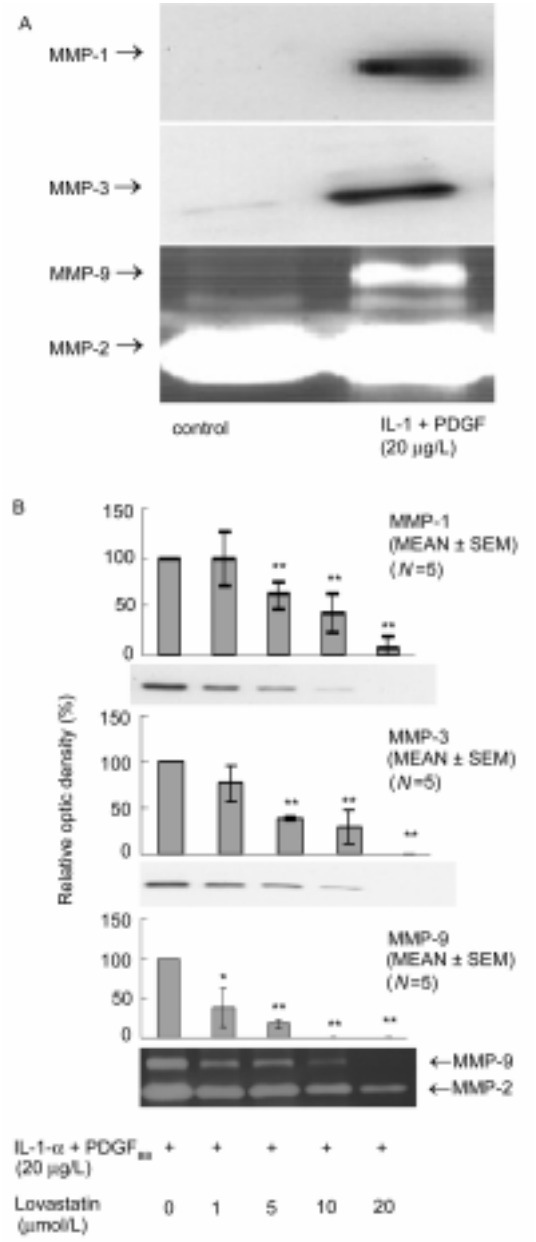

Fig.2 Lovastatin inhibited production of MMP-1, -3 and -9 dose-dependently. Incubation of rabbit $VSMC_S$ with IL-1-α and $PDGF_{BB}$ (20 μg/L, respectively) for 48 h strongly increased production of MMP-1, -3 and -9 (2A). Incubation of $VSMC_S$ with different dose of lovastatin for 48 h in the presence of IL-1-α and $PDGF_{BB}$ reduced production of MMP_S in a dose-dependent manner (2B). All compared with cells stimulated by IL-1-α and $PDGF_{BB}$. $*p<0.05$, $**p<0.01$.

5 Conclusion

Lovastatin inhibited NF-κB activity and the production of MMP-1, -3 and -9 upregulated by IL-1-α and $PDGF_{BB}$ in rabbit VSMCs, which contributes to statins' anti-inflammatory effects independent of cholesterol reduction.

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